UNIVERSIDADE FEDERAL DO PARANÁ

VICTOR AGUIAR DE SOUZA PENHA

ECOLOGIA E MACROEVOLUÇÃO DAS AVES E A RELAÇÃO COM PARASITOS CAUSADORES DE MALÁRIA: IMPLICAÇÕES SOBRE A COLORAÇÃO DE PLUMAGEM E INFLUÊNCIA DA HISTÓRIA DE VIDA

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Orientadora: Profa. Dra. Lilian Tonelli Manica

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“Eu nunca poderia pensar em educação sem amor.
É por isso que me considero um educador: acima
de tudo porque sinto amor.” (Paulo Freire)

RESUMO

Parasitos causadores de malária aviária ocorrem em uma alta diversidade de hospedeiros e podem influenciar muitas características, como a coloração da plumagem. Assim, o presente trabalho teve quatro capítulos, dois macroecológicos e dois estudos locais. O primeiro capítulo estudou a relação entre as características da história de vida das aves e o clima na prevalência de *Plasmodium* e *Parahaemoproteus*, ambos protistas transmitidos por vetores e que causam malária aviária. Amostramos 2.315 indivíduos pertencentes a 47 espécies de traupídeos (Passeriformes: Thraupidae) e rastreamos todos os indivíduos quanto à presença de hemosporídeos. Descobrimos que a prevalência de *Parahaemoproteus* foi maior em espécies que habitam habitats abertos, locais com temperaturas mais baixas e com incubação mais longa. A prevalência de *Plasmodium* foi maior nas espécies que se juntam a bandos mistos e habitam locais mais quentes. O segundo capítulo também utilizou os traupídeos como grupo de estudo para entender como o parasitismo por hemosporídeos afeta a coloração da plumagem. Para isso, amostramos 4.234 indivíduos de 53 espécies de traupídeos e usamos dados públicos e disponíveis para a coloração da plumagem, filogenia das aves e características da história de vida. Descobrimos que o dicromatismo da plumagem foi maior nas espécies com maior prevalência de parasitos causadores de malária. Traupídeos machos e fêmeas com maior complexidade de plumagem tiveram maior prevalência e riqueza de hemosporídeos, respectivamente. Por fim, espécies menores foram mais associadas com o dicromatismo que espécies maiores. No terceiro capítulo estudamos a relação entre coloração da plumagem, condição corporal, corticosterona e hemosporidiose em duas espécies, o pula-pula assobiador (*Myiothlypis leucoblephara*) e o chupa-dente (*Conopophaga lineata*) em uma reserva de Mata Atlântica no sul do Brasil. Encontramos associação positiva entre hemosporidiose e corticosterona de penas para o pula-pula assobiador. Em contrapartida, para o chupa-dente, encontramos que a ocorrência de parasitos explicou negativamente a saturação da coloração de plumagem do ventre dessa espécie. Por fim, como os parasitos raramente ocorrem isoladamente, estudamos o efeito de infecções por hemosporidioses e parasitos intestinais em um estudo de caso usando pintarroxo-caseiro (*Haemorhous mexicanus*) como sistema modelo. Realizamos o estudo em um campus da Arizona State University, Tempe, Arizona, Estados Unidos da América. Descobrimos que indivíduos infectados por *Plasmodium* tiveram maiores níveis de heterófilos / linfócitos (H/L) no sangue, o que indica maior estresse. Indivíduos com maior parasitemia de *Plasmodium* também foram associados com maior índice H/L, sugerindo que não só a infecção, mas a severidade da doença aumenta o H/L. Encontramos também que indivíduos infectados por hemosporídeos tiveram maiores concentrações de luteína circulante. Por outro lado, indivíduos infectados por coccídeos tiveram uma supressão de vitamina E, sugerindo que deve haver uma função de estimulante imune para essa vitamina em aves. Por fim, machos mais com coloração mais avermelhada tiveram maiores concentrações de 3-hidroxi-equinenona, um importante carotenoide para a produção da coloração vermelha em aves. Em resumo, esta tese de doutorado é uma contribuição importante para uma melhor compreensão da relação ave-parasito, em especial a relação intrínseca de hemosporídeos, coloração da plumagem e os hormônios do estresse, além da relação entre hemosporídeos e coccídea em pintarroxo-caseiros.

Palavras-chave: *Plasmodium*, *Parahaemoproteus*, *Isospora*, matiz, carotenoide, melanina.

ABSTRACT

Avian malarial parasites occur in a high taxonomic diversity of hosts, and can influence many traits, such as the plumage coloration. This present work had four chapters, two macroecological and two local study cases. The first chapter studied the relationship between avian life-history traits and climate on the prevalence of *Plasmodium* and *Parahaemoproteus* parasites, vector-borne protists. We sampled 2,315 individuals belonging to 47 tanager species (Passeriformes: Thraupidae) and screened all individuals for the presence of haemosporidian parasites. We found that the *Parahaemoproteus* prevalence was higher in species inhabiting open habitats and in locations with lower temperatures and having a longer incubation. *Plasmodium* prevalence was higher in species joining mixed-species flocks and from hotter locations. The second chapter also used tanagers as the study group to understand how haemosporidian parasites affect the plumage coloration. To this end, we screened the blood samples of 4,234 individuals from 53 tanager species, and used publicly available data for the plumage coloration, bird phylogeny, and life-history traits. We found that the plumage dichromatism was higher in species with an increased prevalence of haemosporidian parasites. Male and female tanagers with higher plumage complexities had higher haemosporidian prevalence and richness, respectively. Lastly, dichromatic species had also a smaller body size, compared to larger species. In the third chapter we studied the relationship between plumage coloration, body condition, corticosterone and haemosporidioses in two species, the white-browed warbler (*Myiothlypis leucoblephara*), and the rufous gnateater (*Conopophaga lineata*) in an Atlantic Forest protected area in south Brazil. We found a significant positive association between hemosporidioses and feather corticosterone for the white-browed warbler. On the other hand, we found that parasites negatively explained the saturation of the plumage coloration in rufous gnateaters. Finally, as the parasites rarely occur in isolation, we studied the effect of haemosporidian and intestinal parasites infections in a case study using house finches (*Haemorhous mexicanus*) as a model system, located at Arizona State University. We found that *Plasmodium*-infected individuals had higher levels of heterophils/lymphocytes (H/L) in their blood, which indicates greater stress. Individuals with higher *Plasmodium* parasitemia were also associated with a higher H/L index, suggesting that not only infection, but the severity of the disease increases H/L. We also found that individuals infected with haemosporidioses also had higher concentrations of circulating lutein. On the other hand, individuals infected with coccidia had a suppression of vitamin E, suggesting an immune stimulant function for this vitamin in birds. Finally, redder males had higher concentrations of 3-hydroxy-echinenone, an important carotenoid for producing red coloration in birds. In summary, this study is an important contribution to the understanding of the bird-parasite relationship, specially and the intrinsic relationship of haemosporidian parasites, plumage coloration and stress hormones, as well as between haemosporidioses and coccidiosis in house finches.

Keywords: *Plasmodium*, *Parahaemoproteus*, *Isospora*, hue, carotenoids, melanin.

Sumário

1.0 Introdução

Parasitos causadores de malária aviária ocorrem em várias espécies (Agosta *et al.*, 2010) de diferentes habitats, de modo que associações parasitárias são comuns em comunidades (Cable *et al.*, 2017). Como parasitos podem afetar negativamente os hospedeiros, entender os padrões de distribuição e ocorrência de parasitos é fundamental para melhor compreender os ciclos de doenças (Ducatez *et al.*, 2020). Por exemplo, parasitos causadores de malária estão associados a indivíduos de espécies de aves com coloração de plumagem menos conspícua (Romano *et al.*, 2019; Penha *et al.*, 2020a), além de reduzirem a condição e o *status* de saúde de hospedeiros (Himmel *et al.*, 2020), podendo até causar a morte (Permin and Juhl, 2002; Atkinson and Samuel, 2010; Jia *et al.*, 2018). Parasitos causadores de malária (Ordem Haemosporidae, gêneros *Plasmodium*, *Haemoproteus*, *Parahaemoproteus* e *Leucocytozoon*) são protistas com duas fases em seu ciclo, uma sexuada, que ocorre nos vetores, e uma assexuada, que ocorre em vertebrados como aves e mamíferos. Cada gênero tem um grupo de vetores específico de dípteros, sendo que mosquitos (Culicidae) são vetores de *Plasmodium*, moscas (Hippoboscidae) são vetores de *Haemoproteus*, maruins (Ceratopogonidae) vetores de *Parahaemoproteus*, e moscas (Simuliidae) de *Leucocytozoon* (Santiago-Alarcon *et al.*, 2012). A exposição aos vetores pode ser influenciada, principalmente, por dois fatores, clima e traços da história de vida de hospedeiros. Maiores temperaturas e precipitações podem criar condições ótimas para o desenvolvimento de vetores, aumentando assim, a prevalência de parasitos causadores de malária em aves (Loiseau *et al.*, 2013). Por outro lado, maiores tamanhos corporais, nidificação e forrageamento mais altos no estrato florestal, participação em bandos da mesma espécie ou mistos e migração podem aumentar as chances de indivíduos se infectarem por malária aviária, por se tratar de condições que aumentem as chances de se encontrar vetores infectados pelo parasito (Garvin and Greiner, 2003; Laporta *et al.*, 2011; Svensson-Coelho *et al.*, 2013; Ibañez-Justicia and Cianci, 2015; Lutz *et al.*, 2015). Ainda que nas últimas décadas tenha havido um crescente entendimento da distribuição espaço-temporal de malária aviária, ainda falta entender como a interação entre parasitos e hospedeiros funciona na região Neotropical, região de alta diversidade de aves e de parasitos.

Uma vez infectadas, aves podem ter o investimento energético desbalanceado, uma vez que há uma tendência de investir mais em resposta imune do que em outras demandas fisiológicas, como coloração de plumagem e reserva energética (Hill *et al.*, 1999). A coloração de plumagem origina-se, primordialmente, de duas fontes: da coloração estrutural ou de pigmentos (Hill and McGraw, 2006). A coloração estrutural é causada pela reflexão diferencial da luz sobre a pena (Maia *et al.*, 2009). Por outro lado, pigmentos, majoritariamente carotenoides e melanina, são provenientes da dieta e do metabolismo do aminoácido, respectivamente (Hill and McGraw, 2006). O carotenoide por exemplo, além de compor a coloração também pode ser utilizado como um estimulante imune, aumentando a imunidade humoral e celular em aves (Hill and McGraw, 2006). Assim, indivíduos infectados por parasitos causadores de malária podem aparecer menos conspícuos, o que pode atrapalhar no processo de escolha por parceiros sexuais em períodos reprodutivos (Hamilton and Zuk, 1982). A maior parte dos estudos tentou compreender como parasitos causadores de malária, seja individualmente ou em nível de comunidade, afetam a coloração da plumagem (Figuerola *et al.*, 1999; Hõrak *et al.*, 2004; Dias *et al.*, 2016; Romano *et al.*, 2019; De La Torre *et al.*, 2020; Penha *et al.*, 2020b). Dessa forma, ainda são escassos os estudos macroecológicos para compreender como o padrão de dicromatismo, ou seja, a diferença de coloração entre machos e fêmeas, é influenciado pelo acometimento de parasitos causadores de malária. Assim, se parceiros escolhem indivíduos com plumagem mais conspícua, podemos esperar as fêmeas de espécies altamente parasitadas devem usar a coloração da plumagem como um indicativo de escolha de parceiros. Desse modo, ainda não temos respostas para perguntas como (a) aves dicromáticas, e que, portanto, estão sob maior pressão de seleção sexual, são mais parasitadas que aves monocromáticas?

A presença do parasitismo em aves pode influenciar, além do metabolismo e da fisiologia da coloração de plumagem, o processo de estresse. O estresse é considerado como qualquer alteração na homeostase de organismo (Romero, 2012). Em aves, a regulação do estresse ocorre, principalmente, pela atuação da corticosterona, um hormônio controlado pelo eixo hipotálamo – glândula pituitária – glândula adrenal. A produção de corticosterona ocorre em momentos de estresse, como privação alimentar, presença de predadores e de parasitos (Bortolotti *et al.*, 2008). Como consequência, o aumento de corticosterona promove mudanças comportamentais para aumentar a probabilidade de sobrevivência dos indivíduos estressados. No entanto, um aumento prolongado deste hormônio pode causar efeitos detrimentais, que pode ser bastante problemático em aves (Butler *et al.*, 2010). Além da corticosterona, há outros parâmetros que servem como uma aproximação a índices de estresse, como a razão entre heterofilos e linfócitos (H/L). Heterófilos são utilizados para combater um amplo espectro de microrganismos (Harmon, 1998), além de ativarem citocinas para aumentar o poder de fagocitose (Kogut *et al.*, 1993). Por outro lado, linfócitos estão relacionados com a defesa humoral, aumentando a produção de anticorpos (Sharma, 1991). Assim, alta H/L é frequentemente relacionada como uma medida de estresse crônico, como um aumento da infecção por parasitos causadores de malária (da Silva Rodrigues *et al.*, 2021). No entanto, estudos que envolvam diversas métricas de condição relacionadas ao estresse, como coloração de plumagem, produção de corticosterona, condição corporal ainda são poucos com espécies de aves selvagens.

O objetivo dessa tese foi estudar a relação entre parasitos causadores de malária e aves, e os resultados são apresentados em quatro capítulos. Os capítulos estão divididos pela escala ecológica, sendo dois macroecológicos e dois estudos locais. Os estudos macroecológicos buscaram elucidar a relação parasito-hospedeiro considerando tanto características do parasito quanto do hospedeiro. O primeiro capítulo teve como objetivo estudar o efeito do clima e dos traços da história de vida de aves da família Thraupidae na prevalência de parasitos causadores de malária (*Plasmodium* e *Parahaemoproteus*). O segundo capítulo estudou os efeitos da alta prevalência e riqueza de linhagens de parasitos causadores de malária no dicromatismo sexual e complexidade de coloração de plumagem de espécies de aves da família Thraupidae. Para o primeiro e o segundo capítulo, utilizei dados de aves capturadas de diversos países da região neotropical, do sul do México até a Argentina. Todos os indivíduos capturados foram testados para a presença de parasitos, gênero *Plasmodium* e *Parahaemoproteus*. Nos dois capítulos, foram feitos modelos filogenéticos generalizados de mínimos quadrados (PGLS – do inglês *phylogenetic generalized least square models*), que levam em consideração a proximidade filogenética entre as espécies para estudar as relações propostas. Já para o terceiro e quartos capítulos foram feitos estudos de caso na Reserva Mananciais da Serra, Piraquara, PR, Brasil e no campus universitário da Arizona State University, Tempe, AZ, Estados Unidos da América. No terceiro capítulo estudei a relação entre a corticosterona presente na pena, ocorrência de parasitos causadores de malária aviária, condição corporal, e coloração de plumagem em duas espécies: pula-pula assobiador (*Myiothlypis leucoblephara,* Passeriformes: Parulidae) e o chupa-dente (*Conopophaga lineata*, Passeriformes: Conopophagidae). Nesse capítulo foi feita uma análise de passos, que permite testar múltiplas relações entre variáveis, já que essas variáveis estudadas podem influenciar-se mutuamente. Por fim, o último estudo foi feito na espécie pintarroxo-caseiro (*Haemorhous mexicanus*, Passeriformes: Fringillidae). Já que parasitos raramente ocorrem em isolamento (Sweeny *et al.*, 2021), esse trabalho estudou os efeitos de infecção por *Plasmodium* e coccidiose em indivíduos de uma população livre por meio da extração de diversas métricas fisiológicas, como o índice H/L, leucócitos globais, condição corporal, carotenoides e vitaminas circulantes. Esse trabalho, então, de uma forma geral contribuiu com uma melhor compreensão nos fatores que influenciam a ocorrência da malária aviária em espécies de traupídeos, além de estudar como a alta prevalência influencia na evolução de traços secundários dessas espécies, indicando os efeitos da seleção sexual. Além disso, estudei a relação entre a ocorrência de parasitos causadores de malária com a corticosterona, e ocorrência de parasitos e coloração de plumagem. Por fim, mostramos como a infecção por dois diferentes parasitos pode ser influenciada por diferentes mecanismos fisiológicos. Dessa forma, esse estudo reforça a recorrente testagem de doenças em aves silvestres como possível identificador de espécies vulneráveis.

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2.0 Capítulo 1: Host life-history traits and climate predict haemosporidian parasite prevalence in tanagers (Aves: Thraupidae)

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Host life-history traits and climate predict haemosporidian parasite prevalence in tanagers (Aves: Thraupidae)

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2.1 ABSTRACT

Vector-borne parasites are important ecological drivers influencing life-history evolution in birds by increasing host mortality or susceptibility to new diseases. Therefore, understanding why vulnerability to disease varies within a host clade is a crucial task for conservation biology and for understanding macroecological life-history patterns. Here, we studied the relationship of avian life-history traits and climate on the prevalence of *Plasmodium* and *Parahaemoproteus* parasites. We sampled 2,315 individual birds belonging to 47 species of the family Thraupidae. Individuals were captured between 2007 and 2018 at 92 locations. We produced two phylogenetic generalized least squares models with *Plasmodium* and *Parahaemoproteus* prevalence as our response variable, and with the following predictor variables: climate PC1, climate PC2, body size, mixed-species flock participation, incubation period, migration, nest height, foraging height, land cover, and diet. We found that *Parahaemoproteus* prevalence was higher in species inhabiting open habitats with lower overall temperature. Tanager species with longer incubation periods had higher *Parahaemoproteus* prevalence as well, and we hypothesize that these longer incubation periods overlap with optimal vector abundance, resulting in a higher probability of infection among adult hosts during their incubation period and among chicks. Lastly, we found that *Plasmodium* prevalence was higher in mixed-species flock participants and in hotter habitats. We discuss the consequences of higher disease prevalence in relation to global climate change and flocking behavior.

Keywords: diet, habitat type, *Parahaemoproteus*, incubation period, *Plasmodium*, temperature.

2.2 INTRODUCTION

Vector-borne haemosporidian parasites can negatively impact host fitness by mediating life-history trade-offs, such as trading investment in immune defense over investment in plumage coloration in response to infection (Hõrak *et al.*, 2001; Delhaye *et al.*, 2018; Penha *et al.*, 2020). Furthermore, haemosporidian infections have been associated with avian mortality (Permin and Juhl, 2002; Atkinson and Samuel, 2010; Jia *et al.*, 2018), and to higher health-related deterioration (Himmel *et al.*, 2021).

Haemosporidian parasites (genera *Plasmodium* and *Parahaemoproteus*) cause malaria and related diseases in wild and domesticated birds; these parasites are ecologically and evolutionarily diverse, with a worldwide distribution (Valkiūnas, 2005; Perkins, 2014). Each haemosporidian genus is transmitted to the avian host by a different group of dipteran vectors; *Plasmodium* by mosquitoes (Culicidae) and *Parahaemoproteus* by biting midges (Ceratopogonidae) (Santiago-Alarcon et al., 2012a). Because avian haemosporidian parasites are broadly distributed, common in avian populations, and easily detected in small blood samples, they provide an important and accessible model system for studying host-parasite interactions.

Within an avian community, host exposure to parasites may be influenced by two major factors: the environment (e.g., climate), and the life-history traits of the host species (Canard et al., 2015; Clark & Clegg, 2017; Lutz et al., 2015; Svensson-Coelho et al., 2014). Climate (particularly rainfall and temperature) may play an important role in parasite exposure through its influence on vector development and abundance (Loiseau *et al.*, 2011; Gehman *et al.*, 2018). For example, in central and west Africa, *Plasmodium* prevalence in the olive sunbird (*Cyanomitra olivacea*) was higher in locations with high temperatures (Sehgal *et al.*, 2011), whereas in community level studies, involving several avian host species, temperature also seems to be a good predictor of *Plasmodium* prevalence, such as in Northeastern Brazil (Rodrigues *et al.*, 2021), and in the Spanish Iberian Peninsula (Illera *et al.*, 2017). However, *Parahaemoproteus* prevalence has shown contrasting results (associated with colder environments) in comparison with *Plasmodium* (Clark, 2018; Clark *et al.*, 2018, 2020), which may be related to the different primary vectors of *Plasmodium* and *Parahaemoproteus* parasites.

Host life-history traits may influence haemosporidian parasite prevalence when these traits are associated with varying host exposure to vectors (Medeiros *et al.*, 2013; Svensson-Coelho *et al.*, 2016). Nesting and foraging height, body size, habitat type, flocking, migratory behavior (Møller and Erritzøe, 1998; Svensson-Coelho *et al.*, 2016), and diet (Lafuma *et al.*, 2001; González *et al.*, 2014; Turcotte *et al.*, 2018; Tchoumbou *et al.*, 2020), are all factors that may influence host exposure to vectors (González et al., 2014; Lutz et al., 2015; Medeiros et al., 2013). For example, mixed-flock participants tend to have a higher haemosporidian parasite prevalence because avian hosts tend to attract more vectors or simple be in contact with more insects (Isaksson et al., 2013), whereas birds foraging and nesting in the canopy and inhabiting closed habitats may have increased parasite prevalence due to a higher vector abundance in these forest strata (Garvin & Greiner, 2003; Ibañez-Justicia & Cianci, 2015; Laporta et al., 2011; Lutz et al., 2015; Swanson et al., 2012; Swanson & Adler, 2010). Host diet may also be an important factor in predicting haemosporidian prevalence, with insectivores harboring higher prevalence, because they seek contact with insects and therefore have increased susceptibility to vectors (Braga *et al.*, 2011; González *et al.*, 2014) and plant-eaters having an adaptive advantage due to the intake of repellent compounds, from the plant matter that they consume, which increases protection against blood-sucking insects (Lafuma *et al.*, 2001). Migration has shown contrasting results, with either migratory host species, due to their higher pathogen exposure (Ciloglu *et al.*, 2020; Anjos *et al.*, 2021; de Angeli Dutra *et al.*, 2021) or resident species exhibiting higher haemosporidian prevalence (Slowinski *et al.*, 2018; Soares *et al.*, 2020), due to increased predictability of hosts to vectors through space and time,. Haemosporidian parasite infection prevalence might also relate to host incubation period (Matthews *et al.*, 2016), which is likely associated with avian life-history trade-offs between immune response and the duration of incubation (Ricklefs, 1992). Therefore, birds with longer incubation periods may have an adaptive advantage by having an increased length of time for B-cell maturation, conferring increased protection against infections (Ricklefs et al., 2018).

Here, we investigated haemosporidian parasite prevalence in tanagers (Passeriformes: Thraupidae), the largest family of songbirds. Tanager species commonly occur from Northern Mexico, through Central America, the Caribbean, and South America, accounting for 12% of bird species in the Neotropical region (Parker III *et al.*, 1996). Tanagers occupy several habitat types, ranging from rainforests to grasslands, with nearly all avian foraging niches being filled by members of the family (Burns *et al.*, 2014). Thraupidae currently includes 377 species placed in 15 subfamilies (Burns *et al.*, 2016; Hilty and Bonan, 2019). Tanager species have a broad range of complex behaviors, habitat preferences, and morphological characteristics (e.g., Beier et al., 2017; Burns et al., 2014; Lima-Rezende & Caparroz, 2016; Macedo et al., 2012; Manica & Marini, 2012; Nogueira et al., 2014). Because of this impressive diversity, the accumulated knowledge on tanager ecology (Shultz and Burns, 2017), and the fact that they have been well sampled within the Neotropical region, making them a good model system for studying the effects of host life history variation and environmental variation on haemosporidian prevalence.

We aimed to understand the relationships between haemosporidian parasite prevalence, tanager life-history traits, and environmental traits. We tested whether species would have higher haemosporidian parasite prevalence due to increased vector exposure associated with (1) nesting and foraging at lower forest strata, (2) inhabiting less forested, warmer locations (for *Plasmodium*, but not for *Parahaemoproteus*), with increased annual precipitation; (3) participating in mixed-species flocks; (4) having an insectivorous diet; and (5) migrating. We also hypothesize that longer incubation periods would be associated with lower parasite prevalence, possibly due to increased time for maturation of the immune system, which would better protect against infection by malarial parasites.

2.3 METHODS

2.3.1 Data Collection

We assembled haemosporidian screening data from 2,315 individual birds belonging to 47 species in the family Thraupidae. Individuals were captured between 2007 and 2018 at 92 locations in seven countries in the Neotropics, including Argentina (Soares *et al.*, 2016), Brazil (Lacorte et al. 2013; Ferreira et al. 2017; Fecchio et al. 2019a, 2021a; Lopes et al. 2020; Penha et al. 2020; Rodrigues et al. 2020), Dominican Republic (Latta and Ricklefs 2010; Soares et al. 2020), Ecuador (Svensson-Coelho *et al.*, 2014), Honduras (this study), Mexico (Fecchio et al. 2019b), and Nicaragua (this study). We could not age and sex most of our species, so we did not consider this in our analysis. All field work was legally permitted in the above countries and was also approved by institutional Animal Care and Use Committees. Molecular protocols, primers used and PCR protocols, can be found in Bell et al. (2015), Lacorte et al. (2013), Latta and Ricklefs (2010), Lopes et al. (2020), Rodrigues et al. (2020), Svensson-Coelho et al. (2014), Soares et al. (2016, 2020), and Penha et al. (2020). In summary, DNA was extracted either by salt precipitation (Ricklefs et al. 2005), phenol-chloroform (Sambrook and Russel 2013), or by using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen, Valencia, CA). DNA extractions were initially screened for the presence of haemosporidian (*Plasmodium* and/or *Haemoproteus*) parasites by amplifying a short fragment of parasite rDNA using either standard (Fallon et al. 2003) or real-time PCR (Bell et al. 2015). Positive samples underwent subsequent nested PCR to amplify the standard barcoding region of the haemosporidian cytochrome *b* gene. Nested PCR was conducted using one of the three following primer sets: HaemF/HaemNR3 and HaemF/HaemR2 (Hellgren et al. 2004), HaemNF/HaemNR2 and HaemF/HaemR2 (Waldenström et al. 2004), or H332F/HaemNR2 and H350F/HaemR2 (Bell et al. 2015). Amplified PCR products were purified and sequenced using either a ABI Prism 377 sequencer. Forward and reverse sequences were assembled using Sequencer v.5.0.1 (Gene Codes Corp., Ann Arbor, MI) or Geneious v.8.1.9 (http://geneious.com; Kearse et al. 2012).

2.3.2 Haemosporidian parasite analysis

To compare lineages identified by our nested PCR protocols to those in the MalAvi database (Bensch *et al.*, 2009), we aligned nucleotide sequences using the program BIOEDIT v. 7.2.0 (Hall, 1999) and verified sequence identities through a local BLAST against the MalAvi database. Lineages identified using the protocol that amplified a longer mtDNA fragment (Ricklefs et al. 2005; Soares et al. 2016, 2020) were successfully matched to known lineages in the MalAvi database only when the two fragments had 100% identical nucleotide sequences in their overlapping region (lineage names here are as in the MalAvi database). Therefore, each successfully matched lineage had a perfect match to only a single MalAvi lineage. Then, we calculated the prevalence of haemosporidian parasites separated in *Parahaemoproteus* and *Plasmodium* for every host species,, as the number of infected individuals divided by the total number of screened individuals. Also, here we are treating *Parahaemoproteus* as a distinct genus from *Haemoproteus* (*Haemoproteus*), following recent phylogenetic advancements in the haemosporidian parasite phylogeny (Martinsen *et al.*, 2008; Borner *et al.*, 2016; Galen *et al.*, 2018). Our haemosporidian parasite prevalence variable was considered including all the sites species were captured in, as well as without a temporal division, since we did not have time of capture for half of our data points. Also, we only took into account the species with at least five screened individuals in our analysis.

2.3.3 Host phylogeny

We used the Thraupidae phylogeny from Burns et al. (2014), which was the first comprehensive tanager phylogeny reconstructed with six molecular markers. Burns et al.'s (2014) phylogenetic hypothesis included new genera that were not within Jetz et al. (2012). This phylogeny produced a highly comprehensive framework for studying macroevolutionary patterns among tanager taxa. We used *ape* (Paradis *et al.*, 2004) to prune out species not in our database from the tree.

2.3.4 Life-history traits and climate

We used the Handbook of the Birds of the World (Hilty and Bonan 2019; https://birdsoftheworld.org/bow/home) to compile the following variables from the 47 tanager species: **body size** (average body length in centimeters); **mixed-flock participation** (participant [frequently or loosely join mixed-flocks] and non-participant [rarely or does not join mixed-species flocks]; **foraging height** (ground [forages on or close to ground]; understory [forages in the midstory of the forest, understory, shrubs or small trees], and canopy [forages in tall trees, or in the canopy of forests]; **migration status** (migrant or resident – complemented with the data from Somenzari et al. (2018) for species that occur in Brazil); and **incubation period** (average number of days). We also collected information on **nest height**, including low (0-1 m / on or close to the ground), middle (1-5 m / in shrubs, small trees, understory, or mid canopy), and high (>5 m or tall trees and upper canopies). We used the data available in Olson & Owens (2005) to categorize foraging ecology including plant eating (herbivore: fruits, seeds, leaves and other plant parts), animal eating (carnivore: arthropods, spiders or others), and a generalist diet (omnivore). We merged Olson & Owens (2005) to categorized diets in only three categories: omnivores, carnivores (mainly insectivores) and herbivores. We used the data available in the Global Habitat Heterogeneity database (GHH - dissimilarity index [12.5 arc-minute / 25 Km]; Tuanmu & Jetz, 2015) as a proxy for habitat type (denoted as the variable name "forest cover" hereafter). We used occurrence data from eBird (https://ebird.org/data/download) and the *extract* function from the *raster* package (Hijmans, 2021), and then averaged GHH values for each species across its distribution. Higher GHH indicates more forested habitats, whereas a lower GHH indicates open habitats. Lastly, we extracted all 19 **climate variables** for the capture sites of all individuals (our 92 different capture sites) from WorldClim 2 (Fick and Hijmans, 2017). For each host species, we averaged climatic values from all sites where a given species was captured. We then performed a principal components analysis to reduce the dimensionality of the climate variables (summary statistics can be found in Supplementary table 1 and Supplementary figure 1). The first and second components together explained 68.7% of the variation and were used as our climatic variables (hereinafter Climate PC1 and PC2). PC1 was primarily related to temperature and was positively associated with variables such as mean annual temperature, minimum temperature of coldest month, and mean temperature of wettest / driest / warmest / coldest quarter, whereas PC2 was positively associated with precipitation variables, such as annual precipitation, precipitation of the driest month, precipitation of the driest quarter, and precipitation of the coldest quarter (Supplementary table 1. Supplemental Figure 3).

2.3.5 Statistical analysis

Using the host phylogeny, we performed two different phylogenetic generalized least square models (PGLS) analyses to test the hypotheses that parasite prevalence is predicted by host-related parameters and climate. We used the *Plasmodium* and

Parahaemoproteus prevalence in separate models as our response variables, with the following explanatory variables: climate PC1, climate PC2, body size, mixed-species flock participation, incubation period, migration, nest height, foraging height, land cover, and diet. Before including all variables, we tested for multicollinearity using the variance inflation factor (VIF) calculated by the *VIF* function from the *regclass* package (James *et al.*, 2014; Petrie, 2020). We used a conservative threshold of two for the values of $\mathrm{GVIF}^{(\frac{1}{2*df})}$ to consider colinear predictors. We found no colinear predictors based on this approach, and all variables were included in the analysis. We tested model convergence with the Ornstein-Uhlenbeck (OU) and Brownian Motion (BM) evolutionary models. We also used a generalized linear model as our model without phylogenetic signal in our comparisons, using the lme4 package, using AIC values. We then selected the best models using an information-theoretic approach (Burnham and Anderson, 2002) with the *dredge* function in the *MuMIn* package (Barton, 2019). When w_i of the best model was below 0.80, we used model averaging in the *model.avg* function in the *MuMIn* package to calculate the model averaged estimates, following the protocol described by Burnham et al. (2011). We assessed the importance of the explanatory variables by evaluating their estimates, unconditional standard errors, and 95% confidence intervals (CI) in the model with the greatest w_i. Since foraging and nest height have three different levels, we used the *relevel* function to change the reference level of each categorical variable to rerun the model and check for a specific pattern of statistically significance. Therefore, we only considered foraging and nest height as significant if a level was different from all other levels. We plotted all significant Variables using the *ggplot2* (Wickham, 2016) package and the predicted values for the variables. All values are presented as mean ± SD, unless otherwise noted.

2.4 RESULTS

2.4.1 Haemosporidian parasites

From the total 2,315 individuals, we found 758 infected with haemosporidian parasites (32% overall prevalence). We found 88 different *Plasmodium* lineages and 64 *Parahaemoproteus* lineages, with *Parahaemoproteus* prevalence marginally higher (17.6%) than *Plasmodium* (15%).

2.4.2 Host life-history traits and climatic variables

We found that most of the tanager species were mixed-species flock participants (80%), non-migratory (87%), middle-forest strata nest builders (59%), and canopy foragers (44%, Figure 1). Host main diet was well-balanced within the species, with 36% herbivores, 34% omnivores and 30% carnivores (Figure 1). Average body size was 14.7 ± 2.8 cm, and incubation period was 13.2 ± 1.1 days. Most of the host species also occurred in more open habitats (Figure 2).

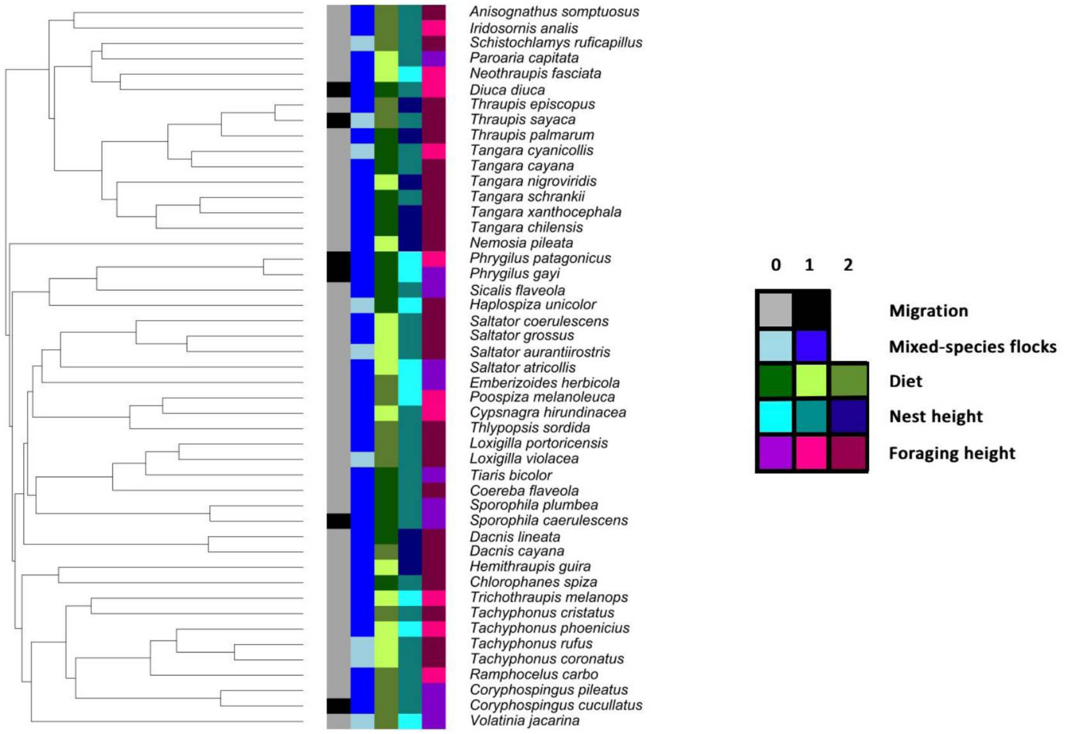

Figure 1: Summary data for categorical life-history variables mapped onto the tips of the trimmed tanager phylogeny, showing as follows: migration (0 – resident; 1 – migrant); mixed-species flocking (0 – non-participant; 1 – participant); diet (0 – plant; 1 – animal; 2 – omnivore); nest height (0 – low; 1 – middle; 2 – high); and foraging height (0 – ground; 1 – understory; 2 – canopy). The colors keys for each category of life-history variables can be seen on the right inset.

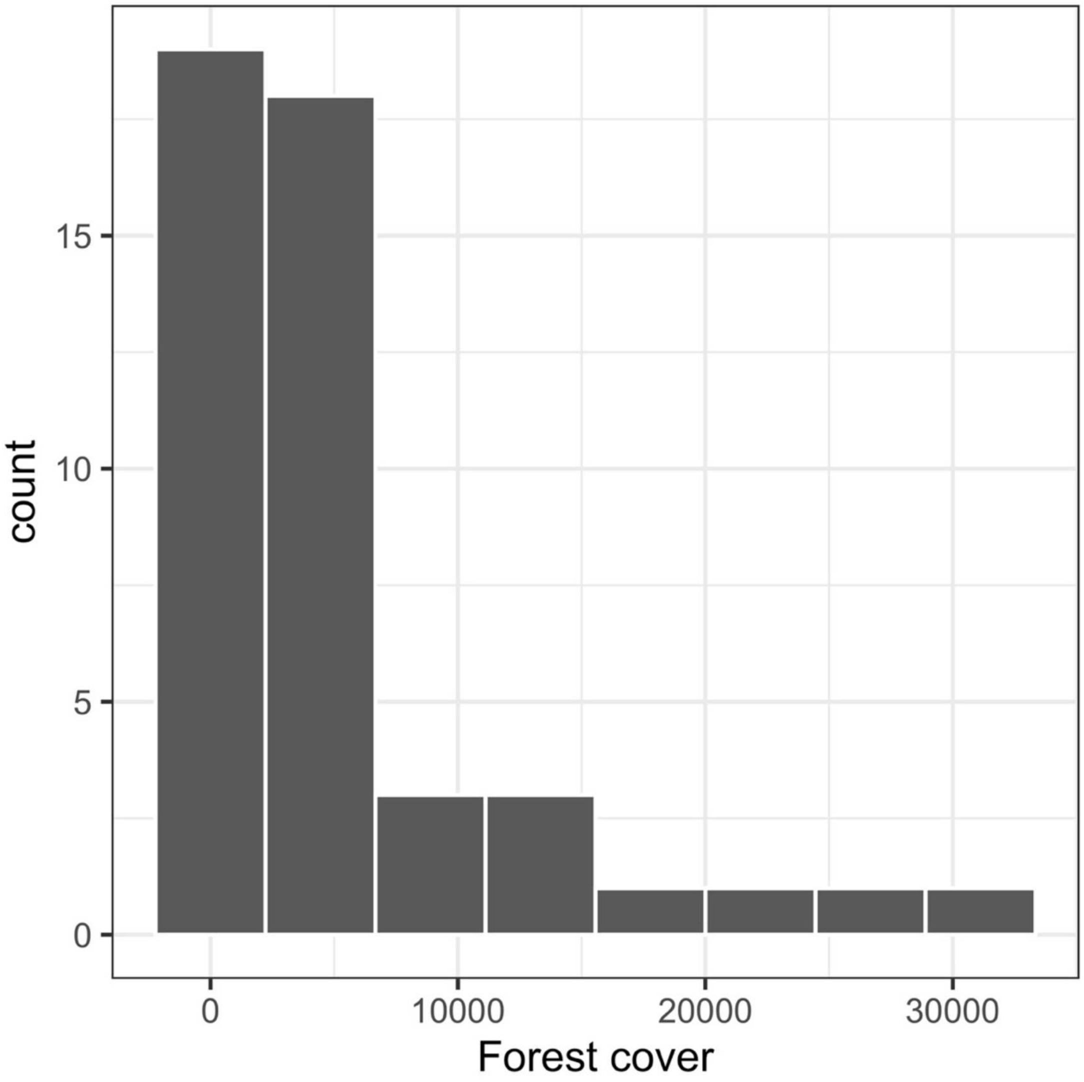

Figure 2: Land cover histogram multiplied by 0.0001, showing that most species inhabit more open habitats (land cover closer to zero indicates less forest coverage). Land cover data retrieved from Global Habitat Heterogeneity – dissimilarity index (https://www.earthenv.org/texture), which contains imagery from Moderate Resolution Imaging Spectroradiometer (MODIS) with pixel values collected from satellite images.

2.4.3 Prevalence models

The best models for *Parahaemoproteus* prevalence are presented in Table 1. We found higher *Parahaemoproteus* prevalence among tanager species inhabiting areas with less forest cover (Table 2, Figure 3), with lower mean annual temperature, lower minimum temperature of coldest month, and lower mean temperature of wettest / driest / warmest / coldest quarter (Table 2, Figure 4), and with longer incubation periods (Table 2, Figure 5).

Table 1: Model selection results of *Parahaemoproteus* and *Plasmodium* prevalence (response variables) and the following explanatory variables: climate PC1, climate PC2, body size, mixed-species flock participation, incubation, migration, nest height, foraging height, land cover, and diet. Variables included in each model are shown together with the models' degrees of freedom, AICc score, delta AIC, and weight (*wi*) are listed below. We only show the models with AIC scores lower than four for *Parahaemoproteus* and *Plasmodium* (complete models can be found in the Supplement material). Results for all 47 sampled tanager species in total. Model comparison using OU (*Parahaemoproteus* model AIC = 82.05; *Plasmodium* model AIC = 132.29), BM (*Parahaemoproteus* model AIC = 141.98; *Plasmodium* model AIC = 133.19), and model without phylogenetic effect (*Parahaemoproteus* model AIC = 139.97; *Plasmodium* model AIC = 140.85), indicated OU as the best in all our models.

Models	df	AICc	**ΔAIC**	w_i
Parahaemoproteus				
Forest cover + Migration + Nest height + Incubation + Climate PC1	9	82.03	0.00	0.419
Forest cover + Migration + Nest height + Body size + Incubation + Climate PC1 + Climate PC2	10	85.09	3.06	0.090
Foraging height + Forest cover + Migration + Nest height + Incubation + Climate PC2	11	85.50	3.47	0.073
Plasmodium				
Diet + Foraging height + Body size + Incubation + Climate PC1 + Climate PC2	11	52.52	0.00	0.777
Diet + Foraging height + Forest cover + Migration + Nest height + Body size + Climate PC1	13	55.02	2.49	0.222

Table 2: Model-averaged estimates, standard errors, and 95% confidence intervals for variables in the model using *Parahaemoproteus* prevalence as the response variable. Significant variables are marked with asterisks. Results for all 47 sampled tanager species in total. Note that for nest height, only two of the combinations were significant (see more information below the table), which means that the variable did not show a statistically significant pattern.

Variables	Estimate	Standard Error	95% C.I.
Intercept[A]	2.15	1.35	-0.52, 4.82
Forest cover	-0.10	0.05	-0.20, 0.00*
Nest height (middle)[B]	0.42	0.16	0.09, 0.74*
Nest height (low)[B]	0.16	0.45	-0.74, 1.07
Incubation	0.50	0.13	0.24, 0.77*
Climate PC1	-0.27	0.07	-0.41, -0.12*
Foraging height (ground)	-0.99	0.51	-2.00, 0.02
Foraging height (understory)	0.20	0.58	-0.94, 1.36
Migration (resident)	-0.37	0.62	-1.59, 0.84
Climate PC2	-0.09	0.17	-0.43, 0.24
Mixed-species flock participation	0.47	0.42	-0.38, 1.33
Body size	-0.01	0.10	-0.22, 0.18
Diet (omnivore)	-0.03	0.41	-0.86, 0.78
Diet (plant)	-0.29	0.36	-1.02, 0.43

[A] Reference level for the categorical variables: diet (animal), foraging height (canopy), migration (migrant), nest height (high), mixed-species flock participation (non-participant).

[B] Changing the reference level to nest height (low): nest height (high): -0.31 ± 0.35 (-0.84, 0.57), nest height (middle): 0.25 ± 0.29 (-0.33, 0.83). Changing the reference level to nest height (middle): nest height (high): -0.42 ± 0.16 (-0.75, -0.09), nest height (low): -0.26 ± 0.36 (-0.97, 0.44).

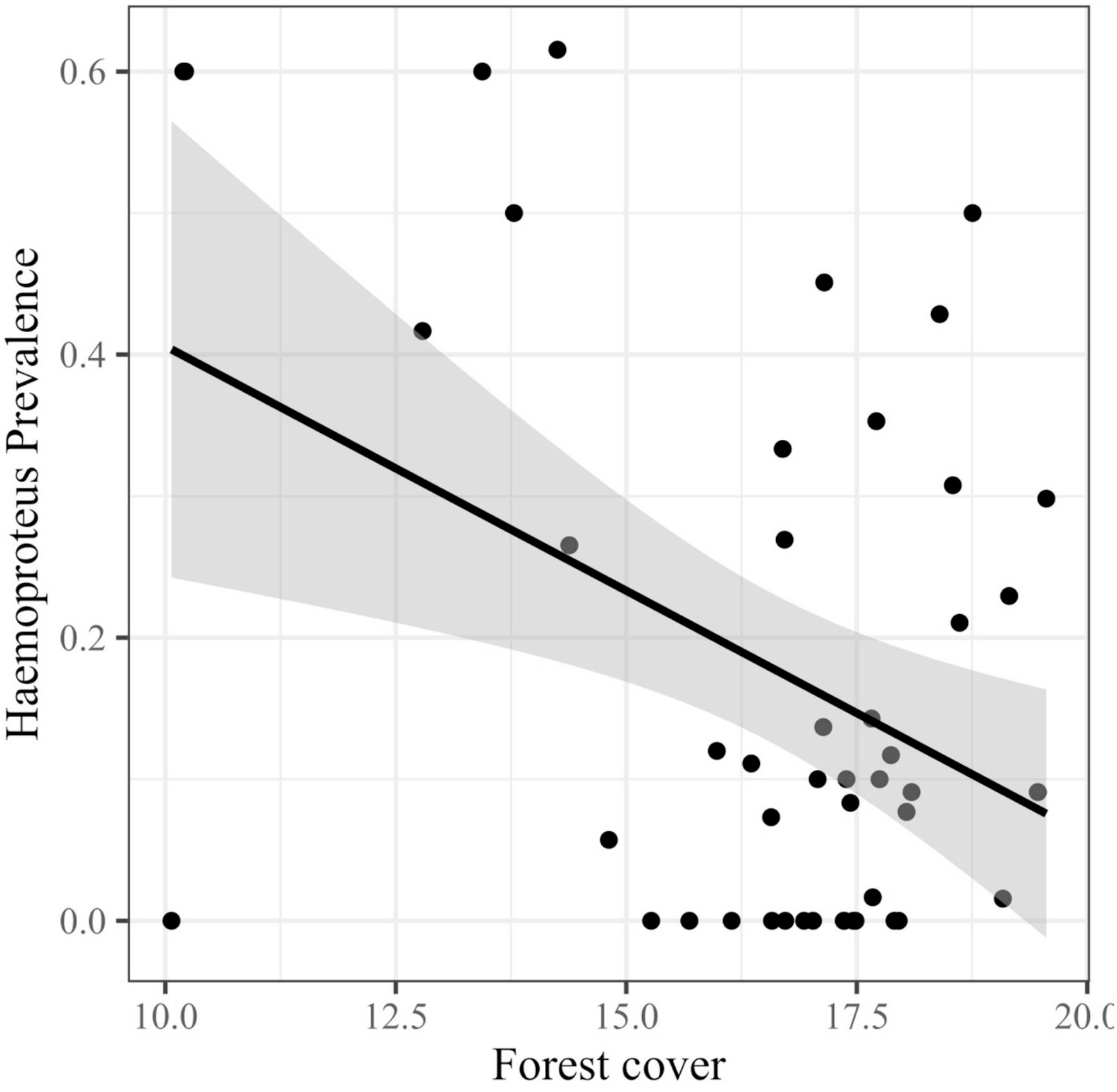

Figure 3: Predicted values of *Parahaemoproteus* prevalence in relation to forest cover at host specimens' locations.

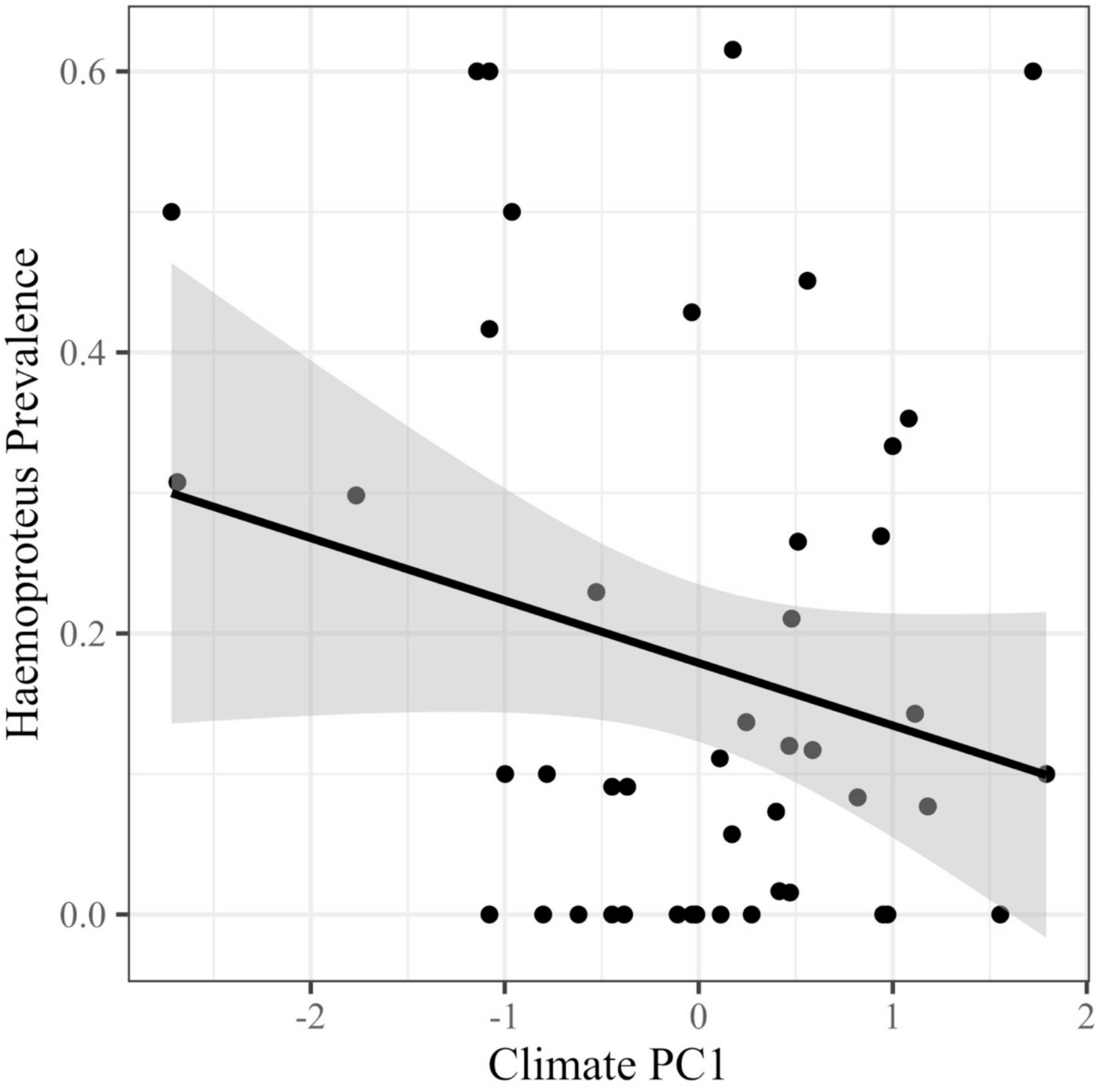

Figure 4: Predicted values of *Parahaemoproteus* prevalence in relation to the climate PC1. Higher climate PC1 indicates higher mean annual temperature, minimum temperature of coldest month, and mean temperatures of the wettest / driest / warmest / coldest quarters.

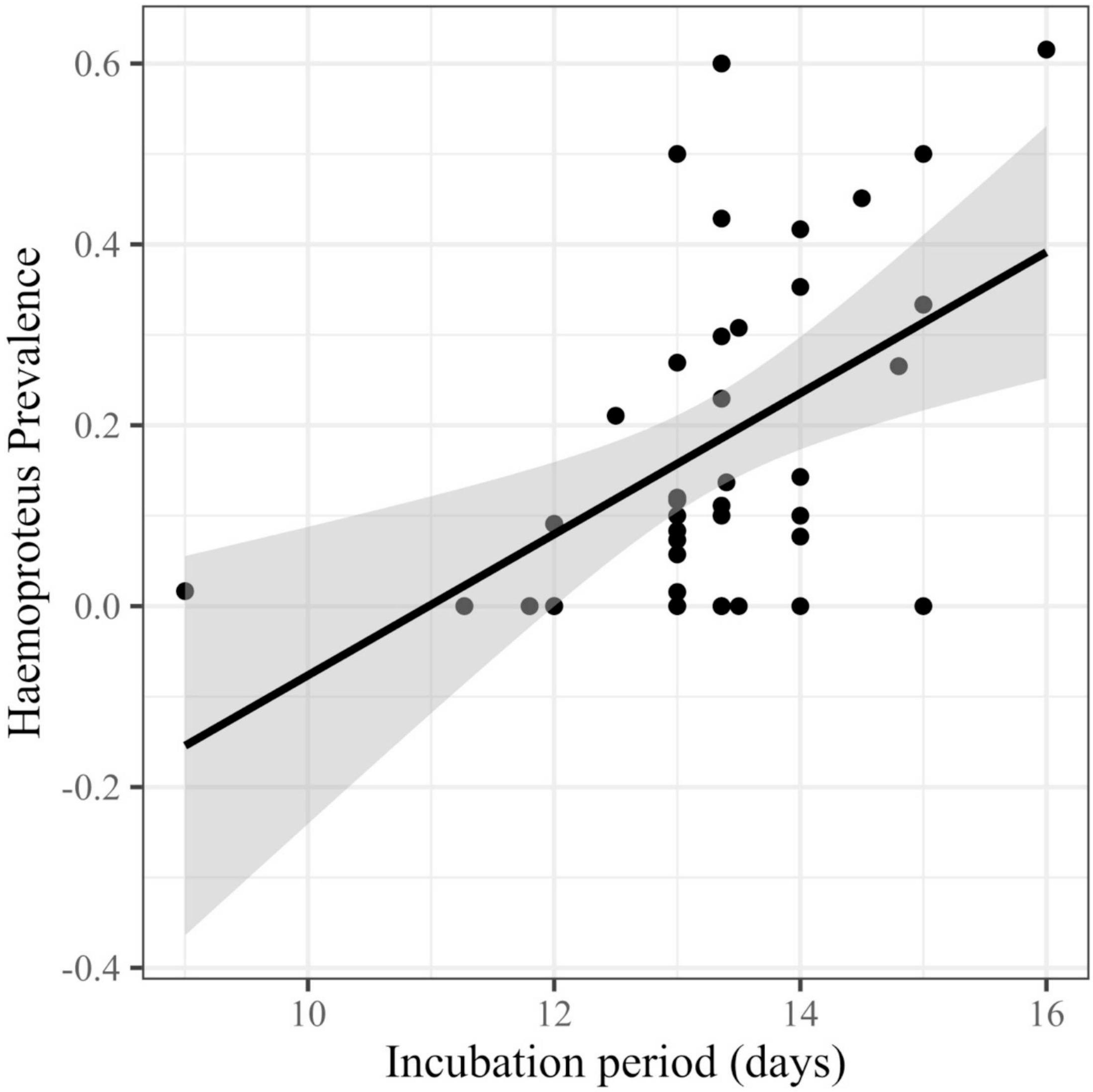

Figure 5: Predicted values of *Parahaemoproteus* prevalence in relation to the incubation period (average number of days).

The best models of *Plasmodium* prevalence are shown in table 1. Tanager species inhabiting locations with higher mean annual temperature, minimum temperature of coldest month, and mean temperature of wettest / driest / warmest / and coldest quarters (Table 3, Figure 6), as well as participating in mixed-species flocks (Table 3, Figure 7), had higher *Plasmodium* prevalence.

Table 3: Model-averaged estimates, standard errors, and 95% confidence intervals of variables in the model using *Plasmodium* prevalence as the response variable. Significant variables are marked with an asterisk. Results for all 47 sampled tanager species in total. Note that for nest height, only two of the combinations were significant (see more information below the table), which means that the variable was not a statistically significant predictor of prevalence.

Variables	Estimate	Standard Error	95% C.I.
Intercept[A]	-0.23	0.49	-1.21, 0.75
Diet (omnivore)	0.45	0.24	-0.03, 0.93
Diet (plant)	-0.18	0.29	-0.74, 0.53
Foraging height (low)	0.12	0.21	-0.33, 0.57
Foraging height (middle)	0.17	0.21	-0.37, 1.03
Body size	-0.12	0.08	-0.29, 0.08
Incubation	0.07	0.06	-0.20, 0.24
Climate PC1	0.27	0.08	0.12, 0.45*
Climate PC2	-0.00	0.07	-0.15, 0.14
Migrant (resident)	-0.39	0.30	-0.96, 0.31
Nest height (low)[B]	-0.34	0.25	-0.36, 0.35
Nest height (middle)[B]	0.38	0.17	0.03, 0.73*
Forest cover	-0.03	0.03	-0.15, 0.02
Mixed-species flock	0.84	0.33	0.16, 1.51*

[A] Reference level for the categorical variables: diet (animal), foraging height (canopy), migration (migrant), nest height (high), mixed-species flock participation (non-participant).

[B] Changing the reference level to nest height (low): nest height (middle): 0.09 ± 0.18 (-0.29, 0.47), nest height (high): 0.51 ± 0.29 (-0.08, 1.11). Changing the reference level to nest height (middle): nest height (low): 0.00 ± 0.17 (-0.35, 0.36), nest height (high): 0.41 ± 0.17 (0.06, 0.76).

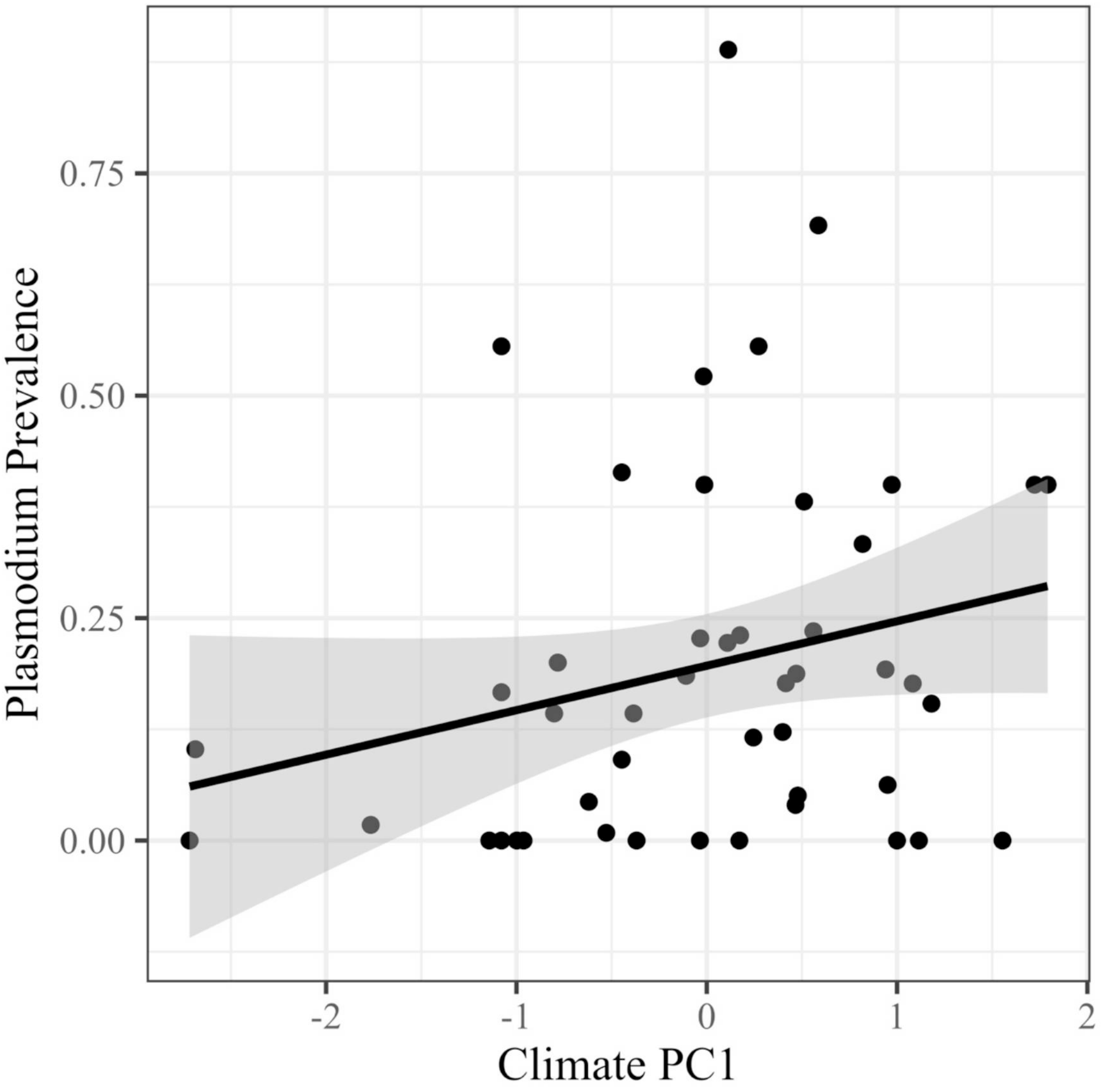

Figure 6: Predicted values of *Plasmodium* prevalence in relation to the climate PC1. Higher climate PC1 indicates higher mean annual temperature, higher minimum temperature of coldest month, and higher mean temperature of the wettest, driest, warmest, and coldest quarters.

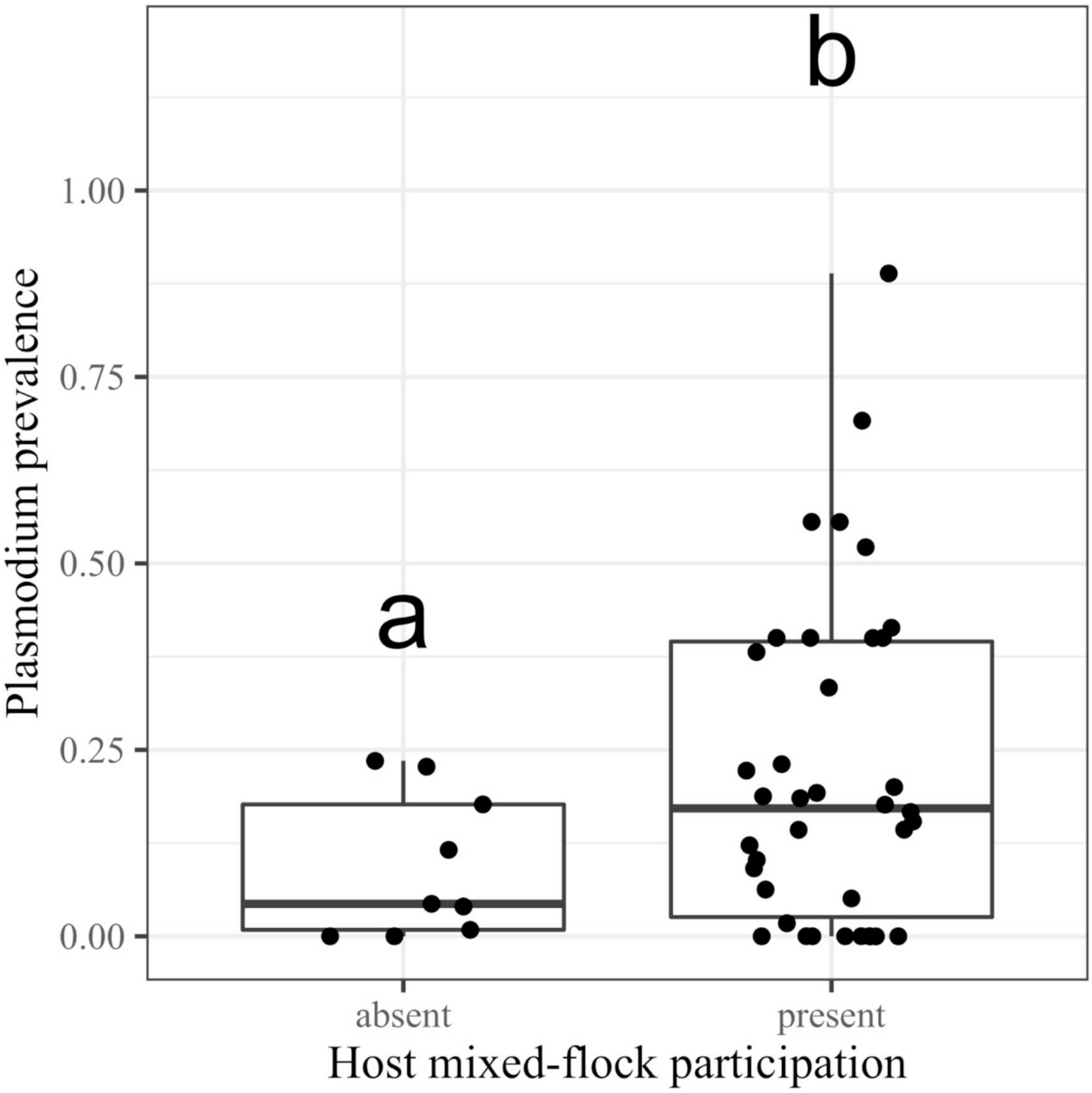

Figure 7: Predicted values of *Plasmodium* prevalence in relation to mixed-species flock participation. Letters above the boxplot indicate statistical significance between mixed-species flock status participation, meaning that tanager species that join mixed-species flocks have higher *Plasmodium* prevalence in comparison with tanager species that do not participate in mixed-species flocks.

2.5 DISCUSSION

Overall, we found that haemosporidian parasite prevalence was explained by both avian life-history traits and climate variables. Specifically, we found that higher *Parahaemoproteus* prevalence was associated with birds occurring in habitats with lower forest cover (more open habitats), colder environments (lower mean annual temperature, minimum temperature of coldest month and mean temperature of wettest / driest / warmest / coldest quarters), and among birds with longer embryonic development. We also found that *Plasmodium* prevalence was more often associated with birds inhabiting warmer locations (higher mean annual temperature, minimum temperature of coldest month, and mean temperature of wettest / driest / warmest / and coldest quarters) and participating in mixed-species flocks.

2.5.1 Climate, life-history traits and *Parahaemoproteus* prevalence

We found, first, that the *Parahaemoproteus* prevalence was higher in tanager species inhabiting locations with lower forest cover (open habitats). Habitat type may be an important predictor of haemosporidian parasite prevalence because it affects the probability of individual birds being exposed to vectors. Previous studies have reported contrasting results relating prevalence to habitat type, either showing higher haemosporidian parasite prevalence in open (Ferreira et al., 2017; Reinoso-Pérez et al., 2016) or in closed habitats (*Leucocytozoon*; Lutz et al., 2015). Biting midges, vectors of *Parahaemoproteus*, are fairly common in nature and have shown some level of host specificity (Martínez-De La Puente et al., 2011; Tomás et al., 2021, but see Bobeva et al., 2015), and these vectors may change their feeding preferences according to the environmental conditions (Santiago-Alarcon *et al.*, 2012b). Biting midges may also occur in different altitudes and habitat types (open x closed) (Möhlmann *et al.*, 2018), which may confer an increased probability of infecting tanagers across our sampling locations. Therefore, our results suggest that tanager species inhabiting places with less forest cover may be more exposed to, or with and increased likelihood of encountering vectors carrying *Parahaemoproteus* parasites, but future studies should identify these vectors as well as their differences across habitat types.

Second, we found that *Parahaemoproteus* prevalence was higher in species inhabiting locations with a lower temperature confirming our predictions. Therefore, because *Parahaemoproteus* are transmitted by biting midges, parasite prevalence may be highly dependent on conditions for vector development. Previous studies have found that either a low (Coral *et al.*, 2015) or a high (Bukauskaite *et al.*, 2015) temperature allow for optimal *Parahaemoproteus* sporogony and vector development. Nevertheless, the optimal temperature for vector larval development is believed to be around 24ºC for biting midges (Van den Eynde *et al.*, 2021), which may relate to the lower threshold of the temperatures found in our study. These contrasting findings emphasize the need for more information on *Parahaemoproteus* sporogony in the Neotropics to better understand the optimal temperature for parasite development and the impact on parasite prevalence in tanagers.

Third, we found that tanager species with a longer incubation period had higher *Parahaemoproteus* prevalence, which was the opposite of what we expected. A longer incubation period is believed to allow for enhanced development of the immune system (Ricklefs, 1992), with higher B-cell maturation, thus conferring better defense against infections (Ricklefs et al., 2018). Therefore, we hypothesize that tanager species facing higher selective pressure from *Parahaemoproteus* parasites may trade investing in reproduction over immunity, producing a weaker immune response to fight-off parasites, and this is supported by other studies. For example, Palacios & Martin (2006) found that longer incubation period does not enhance cellular immune response in several passerine bird species. Alternatively, longer incubation periods may increase the chances of attracting vectors of *Parahaemoproteus parasites*, biting midges (Santiago-Alarcon et al., 2012a), to incubating adults and chicks. Also, longer incubation period is often associated with larger birds and longer nestling periods (Skutch, 1945), which may be highly associated with more frequent or more efficient parasite infection during this period.

2.5.2 Climate, life-history traits and *Plasmodium* prevalence

We found that *Plasmodium* prevalence was higher in species inhabiting locations with higher temperature. Zamora-Vilchis et al. (2012) found that high temperatures and lower elevations favor vector development and increase haemosporidian parasite prevalence in birds. Increased environmental temperature may allow for haemosporidian parasite sporogony in mosquitoes, with decreased temperatures constraining parasite development (Lapointe *et al.*, 2010). For example, low summer temperatures have been linked to reduced sporogony of *Plasmodium* in northern European bird communities (Platonova and Palinauskas, 2021). Our results have important implications for the impact of global warming and its potential effect on malarial parasites, by increasing optimal environmental conditions for vector development and parasite transmission. Therefore, locations prone to have an increase in temperature due to global warming, may create optimal conditions for *Plasmodium* to spread and infect more host species.

Lastly, we found that birds joining mixed-species flocks, either frequently or rarely, had higher *Plasmodium* prevalence. Mixed-species flocks are thought to increase foraging ability or increase surveillance against potential predators (Zou *et al.*, 2018). In the Neotropics, birds often associate in flocks after the breeding season to gain potential benefits from it (Kajiki *et al.*, 2018). However, we show that by joining flocks, there are costs associated with higher infection probability of *Plasmodium* parasites, by (a) increasing visual / olfactory cues associated with vector attraction (Díez-Fernández *et al.*, 2020), or (b) covering a larger spatial area that increases susceptibility to mosquito encounters (Houtan *et al.*, 2006). Our study is in accordance with another in the same region, demonstrating the infection probability costs associated with joining mixed species flocks (González *et al.*, 2014).

In summary, we found important links between temperature and prevalence of *Plasmodium* and *Haemoproteus*. Therefore, our study contributed to a greater understanding of how life-history traits may determine host exposure to parasitic infections. Also, our results aid in identifying host species that may act as potential disease reservoirs and may inform direct conservation efforts towards species that are more vulnerable to haemosporidian parasites.

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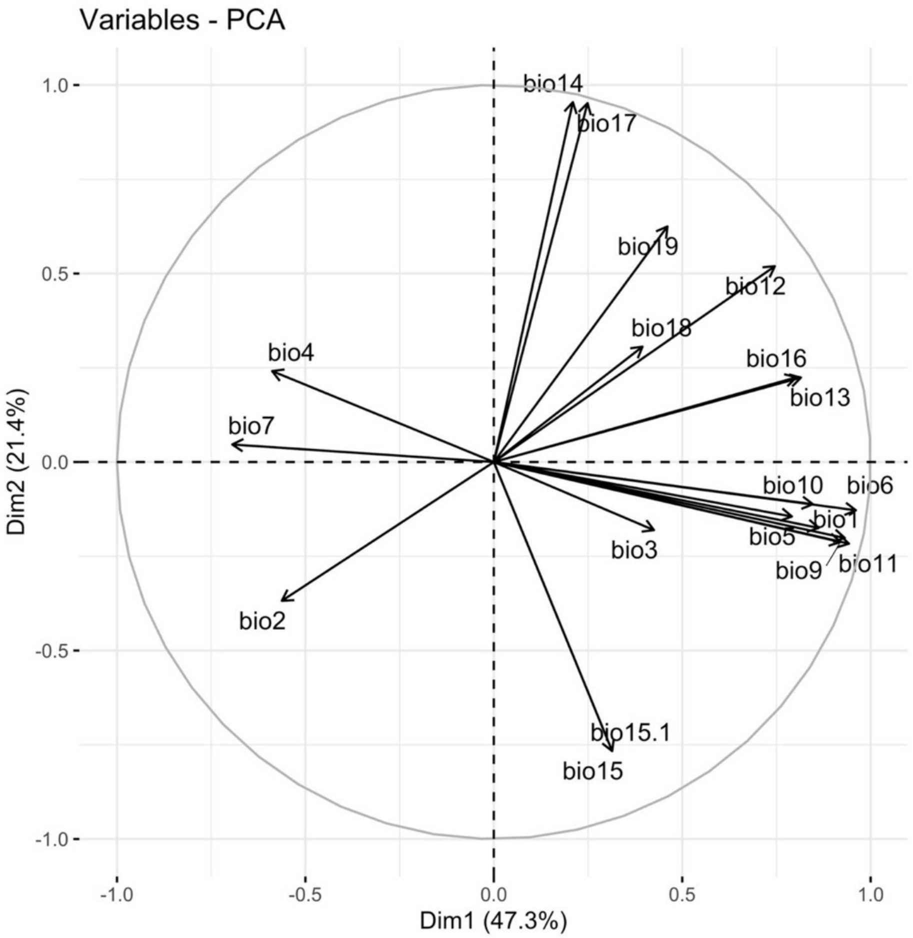
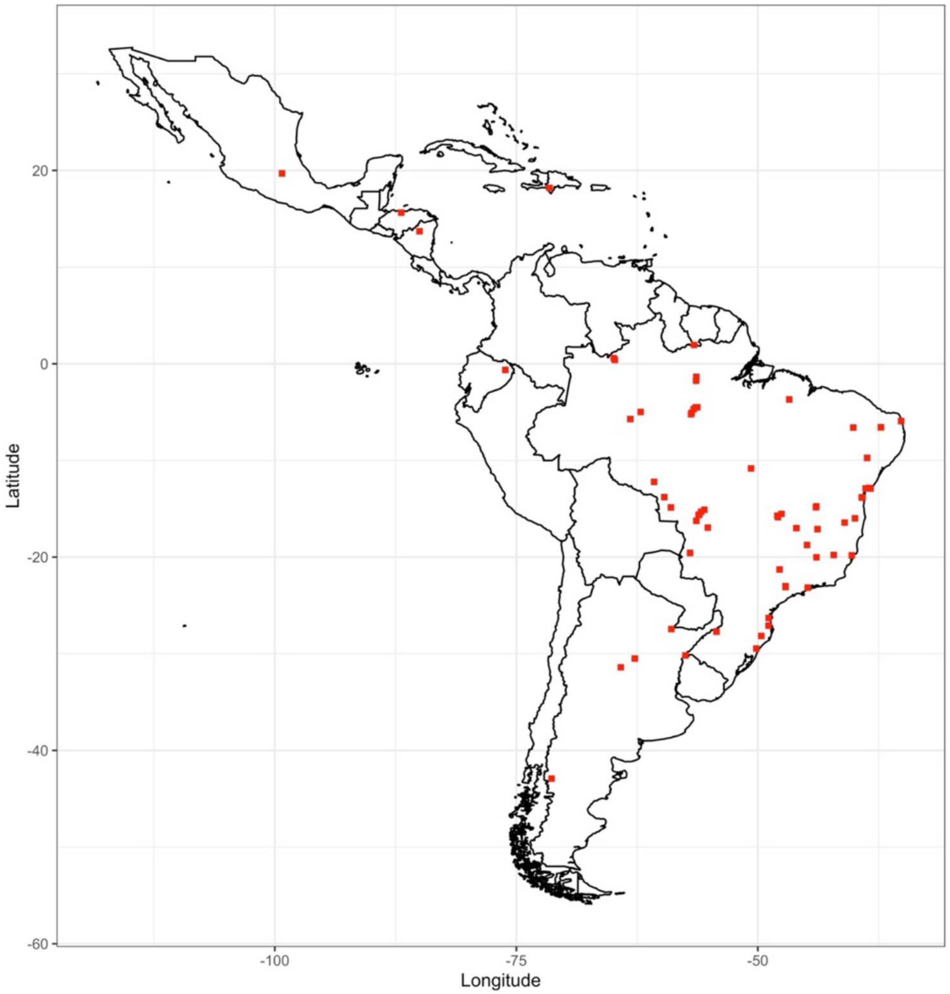
2.7 Supplementary material:

Supplementary table 1: Principal component analysis results for the first and second components, showing their relative important and directionality for PC1 and PC2.

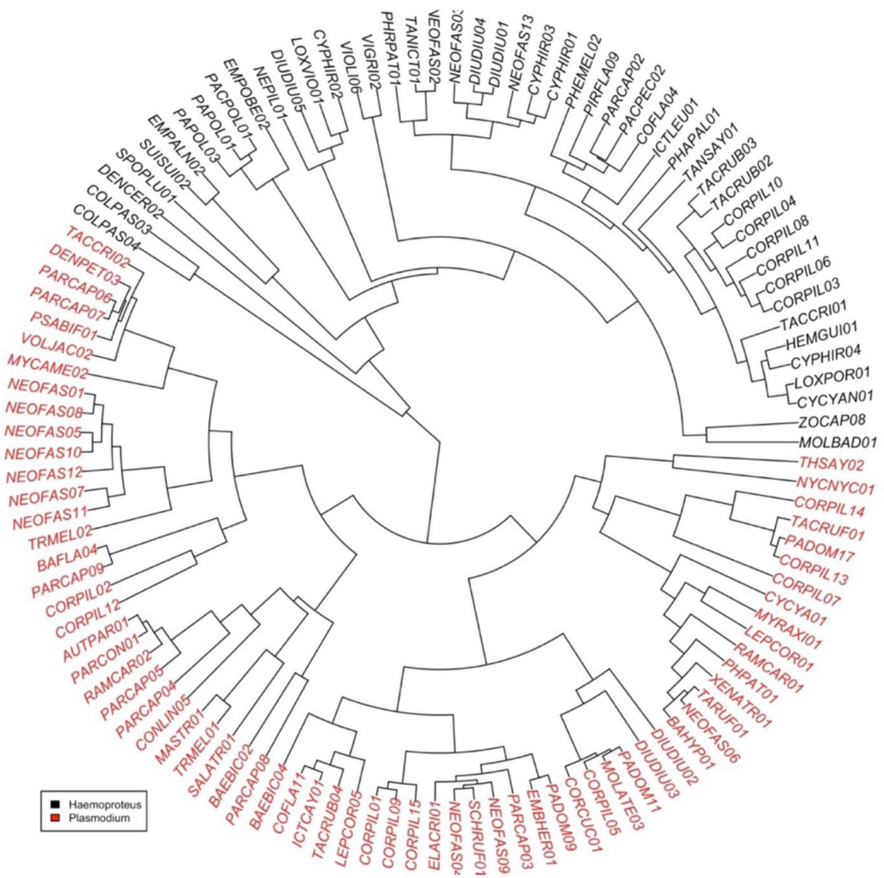
Variables	Variable meaning	PC1	PC2
bio1	Annual mean Temperature	0.30267933	-0.097361
bio2	Mean diurnal range	-0.1827941	-0.1777316
bio3	Isothermality	0.13806491	-0.0872344
bio4	Temperature seasonality	-0.1911351	0.1164353
bio5	Maximum Temperature of warmest month	0.25702738	-0.0698174
bio6	Minimum Temperature of coldest month	0.31219759	-0.0618746
bio7	Temperature annual range	-0.2255817	0.02244019
bio8	Mean Temperature of Wettest quarter	0.28009905	-0.0844349
bio9	Mean Temperature of driest quarter	0.29894555	-0.1025735
bio10	Mean Temperature of warmest quarter	0.27509977	-0.0540146
bio11	Mean Temperature of coldest quarter	0.30626256	-0.1045826
bio12	Annual precipitation	0.24231144	0.25078462
bio13	Precipitation of wettest month	0.26484049	0.10814007
bio14	Precipitation of driest month	0.06812081	0.46090038
bio15	Precipitation seasonality	0.10201755	-0.3702065
bio16	Precipitation of wettest quarter	0.26071112	0.10845269
bio17	Precipitation of driest quarter	0.08081085	0.45965353
bio18	Precipitation of warmest quarter	0.12820231	0.14777211
bio19	Precipitation of coldest quarter	0.14970469	0.30174671



Supplementary figure 1: Biplot of Principal component analysis with all 19 climate variables from WorldClim 2 (Fick & Hijmans, 2017). Here we show the first (x-axis) and second (y-axis) components along with their proportion of explained variability (in parenthesis).



Supplement figure 2: Data occurrences throughout the Americas, ranging from Mexico to southern South America.



Supplement figure 3: Bayesian maximum Clade Credibility Tree for the 167 haemosporidian lineages found in 2,986 individuals belonging to 80 Thraupidae species throughout 73 locations in the Americas.

3.0 Capítulo 2: Haemosporidian parasites and body length influence plumage coloration in tanagers (Passeriformes: Thraupidae)

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Haemosporidian parasites and body length influence plumage coloration in tanagers (Passeriformes: Thraupidae)

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3.1 ABSTRACT

Birds are highly visually oriented and use their plumage coloration as an important signaling trait in social communication. Because males tend to have more complex plumages, sexual dichromatism is usually attributed to female choice. However, plumage coloration is partly condition-dependent, therefore other selective pressures affecting individuals' success may also drive the evolution of this trait. Here we used tanagers as model organisms to study the effects of parasitism by haemosporidian parasites and investment in reproduction in the plumage dichromatism. We screened blood samples of 4234 avian hosts belonging to 53 tanager species for detecting haemosporidian parasites. We used publicly available data for plumage coloration, bird phylogeny, and life-history traits to run three phylogenetic generalized least-square models of plumage dichromatism and complexity. We found that plumage dichromatism was more pronounced in bird species with a higher prevalence of haemosporidian parasites. Also, we found that males and females with a higher plumage complexity had a higher haemosporidian parasite prevalence and haemosporidian parasite lineage richness, respectively. Lastly, smaller species were highly associated to dichromatism, compared to larger species. Our results suggest that haemosporidian parasites shape plumage coloration evolution and may influence the decision-making process of mate choice.

Keywords: sexual dichromatism, sexual selection, female ornamentation, parasite prevalence, plumage coloration complexity, *Plasmodium*, *Parahaemoproteus*.

3.2 INTRODUCTION

Plumage coloration is an important signaling trait in birds, because they are highly visually-oriented organisms (Espmark et al. 2000). Males usually exhibit different patterns of plumage coloration compared to females (Dale et al. 2015), indicating that sexual selection may be an important force generating coloration differences in bird species (Hill and McGraw 2006). According to the female choice mechanism of sexual selection, males in improved condition are better able to compete against other males and to attract mates (Darwin 1871). In house finches (*Haemorhous mexicanus*), for example, more brightly-colored males initiated reproduction earlier and produced more offspring

(McGraw et al. 2001), thus impacting their demographically important reproductive success (Siefferman and Hill 2005).

Sexual dichromatism may also evolve in response to the intensity of sexual selection in species, which may depend upon parameters such as body size and life-history traits. In birds, sexual dichromatism is inversely associated with body size, such that sexual selection appears to be stronger in smaller species (Carballo et al. 2020). This is the case of smaller species of parrots, for example, and is likely due to shorter pair bond duration and increased mate turnover throughout the life-span (Toft and Wright 2015). Therefore, larger species tend to be monochromatic, with both sexes displaying either conspicuous or dull coloration (Carballo et al. 2020). Life-history traits are important predictors of plumage coloration because they reflect patterns of survival and reproduction and, thus, individuals' ability to attract mates. For instance, investment in reproduction may influence plumage coloration in birds as species incubating their clutches for longer periods or incubating more eggs may face a trade-off between color investment and reproductive output (Hasegawa & Arai, 2016; Morrison et al., 2014) In carduelis finches, a clade comprising 125 different species, melanin plumage complexity increases with decreasing clutch size and incubation period (Bókony and Liker 2005).

Perhaps one of the strongest hypotheses to explain plumage coloration diversity in avian species is related to parasitism. Plumage coloration has been previously associated as possibly signaling the health status of organisms, because it may be an indicative of genetic resistance to parasites (Hamilton and Zuk 1982). Plumage coloration pigments, such as carotenoids, are also immune stimulators, meaning that an overuse of carotenoids in plumage coloration may compromise other physiological functions associated with the immune system (Hill et al. 1999), and therefore carotenoid deposition may be an honest signal for condition. Intensely parasitized individuals of house finches usually have dull plumage as a result of an energetic imbalance between investing in plumage coloration and mounting an immune response against parasites (Hill et al. 2004). Also, parasites that do not directly reduce circulating carotenoids, such as haemosporidian parasites, might depress the utilization of this pigment (Hill et al. 2004). For example, plumage coloration saturation and carotenoid chroma predict the probability of an individual being parasitized by haemosporidian parasites, and differences between parasitized and non-parasitized individuals is greater in sexually dimorphic species (Figuerola et al. 1999; Penha et al. 2020). Haemosporidians (Order Haemosporidae, genera *Plasmodium* and *Parahaemoproteus*) are vector-borne protozoans that infect avian blood cells and other tissues for reproduction. Since they are distributed worldwide and parasitize almost all avian families (Valkiunas 2005) with different degrees of dichromatism, these malarial pathogens present a good study system to understand the relationships between avian hosts coloration and their pathogens.

Tanagers (Passeriformes: Thraupidae) are songbirds with diverse life-history traits and elaborate secondary sexual characteristics, notably the plumage coloration and complex songs. This is one of largest avian families and for which these sexual traits have been focus of important macroevolutionary studies (e.g., Mason et al 2014, Shultz and Burns 2017, Drury et al. 2018). Since tanagers are hosts of several haemosporidian parasite lineages (Fecchio et al., 2019a, 2019b, 2020, Rodrigues et al. 2021), they provide an interesting opportunity for studying the relationship between color and parasitism in birds. Also, dichromatism is widespread in the family, occurring at some level in 97% of the species (Burns et al. 2012), and both sexes have complex plumage coloration, but it is greater in males (Shultz and Burns 2017). In a study comprising 351 species of the family, dichromatism was shown to be more influenced by evolutionary changes in males than in females, and to be more correlated with male than female plumage complexity (Shultz and Burns 2017). The importance of life-history traits evolution of plumage coloration in tanagers is also dependent on the light environment, with species showing brighter plumage in open than closed habitats (Shultz and Burns 2017). Despite these recent advances in the tanagers' literature, the impact of haemosporidioses in the dichromatism and coloration complexity in species of this family remains unknown. Here we aim to unravel this host-parasite relationship as well as to make progress to the understanding of how life-history traits influence the evolution of plumage coloration in tanagers. Specifically, we tested whether dichromatism and plumage complexity were negatively related to haemosporidian parasite prevalence and lineage richness, species clutch size, incubation period and body length.

3.3 METHODS

3.3.1 Data collection

We used 4234 individuals from 53 Thraupidae species collected between 2007 and 2018. Each species included in the study was represented by at least 5 captured individuals. Unfortunately, we could not sex and age most of our captured individuals, so we did not include this information in our analysis. Samples included in the study came from eight countries and 92 locations, including Argentina (Soares et al. 2016; Fecchio et al. 2019a), Brazil (Lacorte et al. 2013; Ferreira et al. 2017; Fecchio et al. 2019a, 2021; Lopes et al. 2020; Penha et al. 2020; Rodrigues et al. 2020), Dominican Republic (Latta and Ricklefs 2010; Soares et al. 2020), Ecuador (Svensson-Coelho et al. 2014), Honduras (novel data), Mexico (Fecchio et al. 2019b), Nicaragua (novel data) and Peru (Fecchio et al. 2019a). We either banded all individuals and extracted a blood sample or collected blood samples from host specimens. Then we screened these individual samples for the presence of haemosporidian parasites. All field work followed each country's data collection laws, under specific licenses (Ethics Committee in Animal Experimentation from Universidade Federal de Minas Gerais, Brazil - 254/2011, Universidade de Brasília, Brazil – 129022/2015; Instituto Chico Mendes de Conservação da Biodiversidade: 42578, 3964-7, 33206-1; Centro Nacional de Pesquisa e Conservação de Aves - CEMAVE: 3856, 3239; American Ornithologist's Union and University of North Dakota Animal Care and Use Committee guidelines, Project number 1402-1). Some individuals were collected and deposited after analysis in Instituto Nacional de Pesquisas Amazônicas, Museu Paraense Emílio Goeldi, Field Museum of Natural History, Museo de Zoología Alfonso L. Herrera and The Academy of Natural Science of Drexel University.

3.3.2 Haemosporidian lineage identification

We extracted DNA following the protocols described by Ricklefs et al. (2005) and Sambrook and Russel (2013), or using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen, Valencia, CA). We screened DNA samples for the presence of *Parahaemoproteus* or *Plasmodium*. Specific molecular protocols can be found in Latta and Ricklefs (2010); Lacorte et al. (2013); Svensson-Coelho et al. (2014); Bell et al. (2015); Soares et al. (2016, 2020); Lopes et al. (2020); Penha et al. (2020); and Rodrigues et al. (2020). Briefly, we amplified a standard barcoding region from the cytochrome *b* gene from haemosporidian parasites using nested PCR, and then we sequenced the lineages. We used BIOEDIT v. 7.2.0 (Hall 1999) to align sequences and compare them with the MalAvi database (Bensch et al. 2009) to identify haemosporidian genetic lineages. As our protocols amplified two different regions of the cytochrome b gene, we compared longer mtDNA fragments (Ricklefs et al. 2005; Soares et al. 2016, 2020) through a local BLAST on the MalAvi database (Bensch et al. 2009). We only considered their identities once there was 100% overlap between the fragment and the MalAvi lineage, which was the case for all of the lineages we found. Therefore, each successfully matched lineage had a perfect match to only a single MalAvi lineage. Then, we calculated "lineage richness" of a host species as the total number of lineages found in a given host species, divided by the total number of screened individuals per host species to account for uneven sampling. Finally, we calculated the haemosporidian (*Plasmodium* and *Parahaemoproteus*) parasite prevalence as the number of infected individuals divided by the total number of screened individuals in every species. Also, here we are treating *Parahaemoproteus* as a distinct genus from *Haemoproteus* (*Haemoproteus*), following recent phylogenetic advancements in the haemosporidian parasite phylogeny (Martinsen *et al.*, 2008; Borner *et al.*, 2016; Galen *et al.*, 2018).

3.3.3 Host phylogeny and life-history traits

We used the tanager phylogeny from Burns et al. (2014) and the *drop.tip* function from the *ape* package (Paradis et al. 2004) in R software to prune the tree to the 53 species from our database. We used the Handbook of the Birds of the World Alive (https://www.hbw.com; Hilty and Bonan 2019) to extract the body length, the incubation period (in days) and clutch size (number of laid eggs) for all tanager species.

3.3.4 Plumage coloration

To assess male and female plumage coloration complexity and dichromatism, we used the data from Shultz and Burns (2017), which used data collected from museum specimens, for the avian species represented in our dataset. In summary, Shultz and Burns (2017) used the spectrophotometric measurements to generate a reflectance tetrahedral color space (Stoddard and Prum 2008). Then, they produced information on maximum, average and variance of color span (the Euclidian distance among points inside the tetrahedron), color volume (total volume from the polygon connecting all points in the tetrahedron), maximum, average and hue disparity (differences in angles from the vectors within the tetrahedron), average chroma (average distance between achromatic center and a data point inside the tetrahedral for all members of a given species) and average brilliance (average reflectance). All these variables were referred to as whole-plumage tetrahedral color space (WPTCS) measurements. We used the average whole-plumage color span as our dichromatism measurement (dichromatism from herein). Higher values of dichromatism (i.e., larger distances among points in the tetrahedron) mean that males and females have increased differences in their plumage coloration patterns. We used Shultz and Burns (2017)'s PC1 axis from a principal component analysis including all WPTCS measurements for males and females as our plumage complexity measurement per sex (male and female plumage complexity from herein). Positive PC1 values (with reversed sign to facilitate interpretation) indicate higher values of all WPTCS measurements, suggestive of a more complex plumage, higher contrast among plumage sites, and with larger regions of WPTCS (Shultz and Burns, 2017). In our dataset range, the dichromatism ranged from 0.016 to 0.406, whereas the range of Male PC1 was -6.368 (low complexity) to 5.982 (high complexity), and for Female PC1, from -4.506 (low complexity) to 6.574 (high complexity).

3.3.5 Statistical analysis

To test whether sexual dichromatism is related to haemosporidian parasitism and life-history traits, we built a phylogenetic generalized least square (PGLS) model including dichromatism as response variable, and parasite lineage richness, haemosporidian parasite prevalence, clutch size, incubation period and body length as explanatory variables. We also built two additional PGLS models, one for each sex, to test the relationship between plumage complexity, our response variables, and parasitism (haemosporidian parasite prevalence and parasite lineage richness), clutch size, incubation period, clutch size, and body length, as our explanatory variables.

We tested for absence of multicollinearity with the variance inflation factor (VIF), using the *VIF* function from the *regclass* package (James et al. 2014) for all models. We used a conservative threshold of two for $\mathrm{GVIF}^{(1/(2*df))}$ to consider collinear predictors. We built both Ornstein-Uhlenbeck (OU) and Brownian Motion (BM) PGLS and used the Akaike Information Criterion (AIC) values to test for model fit. We corrected the explanatory and response variables using logarithmic or square-root functions in R, to normalize the distributions whenever necessary, as well as scaled all numeric variables using the *scale* function to keep all variables comparable. We used an information-theoretic approach (Burnham and Anderson 2002) to test the importance of the explanatory variables. We used the *dredge* function from the *MuMIn* package (Barton 2019) to generate all possible models with the explanatory variables. We used model averaging with the *model.avg* function from the *MuMIn* package to calculate the model averaged estimates (Burnham et al. 2011) for all models with delta AIC <4. We selected the most important explanatory variables by assessing the estimate, conditional standard errors, and 95% confidence interval (CI). We used the predicted values from all models to plot statistically significant variables, using the *predict.pgls* function from the *caper* package (Orme et al. 2018). All analysis were performed in R software version 2019 (R Core Team 2019).

3.4 RESULTS

In our sampled species (n = 53), mean ± sd body size was 14.66 ± 2.91 cm, mean ± sd clutch size was 2.5 ± 0.6 eggs, and mean ± sd incubation period was 13.40 ± 1.05 days. We found 149 haemosporidian lineages, 62 *Parahaemoproteus* and 87

Plasmodium, in 1387 individuals tanagers, with 32% overall prevalence. Bananaquit (*Coereba flaveola*) and red pileated finch (*Coryphospingus pileatus*) had the greatest parasite lineage richness (11 lineages each), whereas yellow cardinal (*Gubernatrix cristata*) had the highest haemosporidian parasite prevalence, with all captured individuals being infected.

3.4.1 Plumage dichromatism

We found that more dichromatic tanager species had higher haemosporidian parasite prevalence and smaller body length (Table 1, Table 2, Figure 1). Parasite lineage richness, clutch size and incubation period were not important variables in the model selection (Table 1, Table 2).

Table 1: Results for the dichromatism model selection and the following explanatory variables: haemosporidian parasite prevalence, parasite lineage richness, clutch size, incubation period, and host body length. "+" indicates the presence of variables in each model. Degrees of freedom (df), AICc values, delta AICc, and weight (*wi*) are presented. Here we show the models with delta AICc lower than four. Ornstein-Uhlenbeck (OU) had the lowest AIC and was used as the evolutionary model (Brownian motion [BM] AIC = 142.93; Ornstein-Uhlenbeck [OU] = 139.42).

Explanatory variables								
Prevalence	Lin. richness	Clutch size	Inc. period	Body length	df	AICc	ΔAIC	*wi*
+				+	5	137.98	0.00	0.184
+			+	+	6	138.64	0.66	0.132
+	+			+	6	139.47	1.48	0.087
+		+		+	6	139.90	1.91	0.070
+			+		5	139.99	2.01	0.067
+	+		+	+	7	140.42	2.43	0.054
+		+	+	+	7	140.47	2.49	0.052
+					4	140.94	2.96	0.041
	+			+	5	141.09	3.11	0.038
+		+	+		6	141.27	3.28	0.035
+	+		+		6	141.69	3.70	0.028
+	+	+		+	7	141.81	3.83	0.027

Table 2: Model-averaged estimates, standard errors, and 95% confidence intervals for variables in models of dichromatism. Significant variables are marked with an asterisk.

Variables	Estimate	Standard Error	95% C.I.
Intercept	0.14	0.37	-0.60, 0.89
Haemosporidian parasite prevalence	0.33	0.13	0.06, 0.60*
Lineage richness	0.15	0.14	-0.12, 0.43
Clutch size	-0.14	0.19	-0.53, 0.24
Incubation	-0.17	0.12	-0.41, 0.07
Body length	-0.28	0.13	-0.55, -0.01*

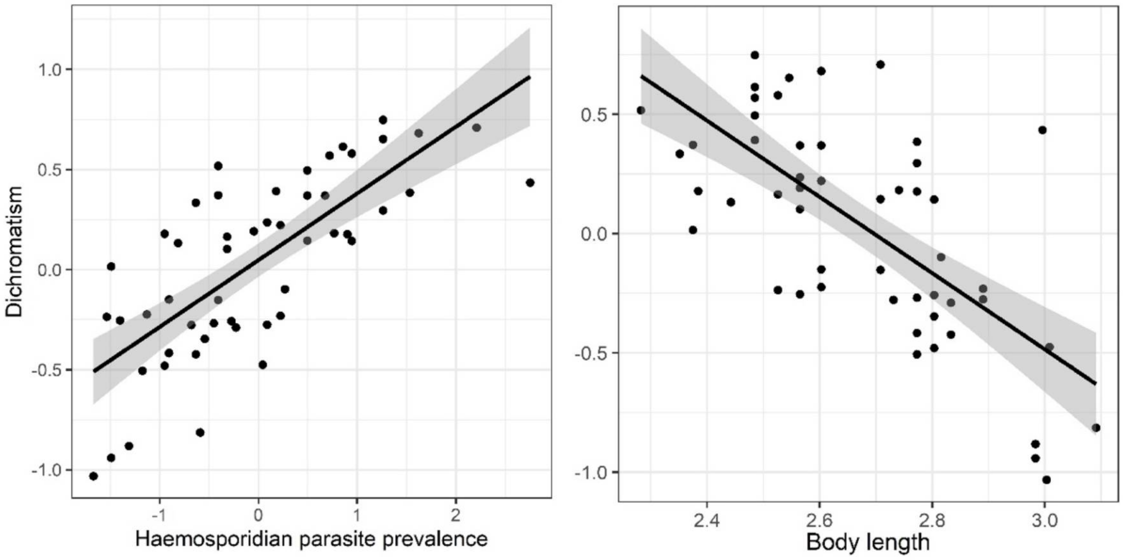

Figure 1: Dichromatism in relation to haemosporidian parasite prevalence (left) and body length (right).

3.4.2 Male and female plumage complexities

The best models of female and male plumage coloration are included in Table 3. We found that more complex male and female plumages were associated with species having a higher haemosporidian parasite prevalence (Table 4, Figure 2) and higher haemosporidian parasite lineage richness (Table 4, Figure 3), respectively.

Table 3: Results for the model selection of male and female plumage complexities and parasite prevalence, parasite lineage richness, clutch size, incubation period, and body length. "+" indicates the presence of variables in each model. Degrees of freedom (df), AICc, delta AICc, and weight (*wi*) are presented. Here we show the models with delta AIC lower than four. Ornstein-Uhlenbeck (OU) had the lowest AIC and was used as the evolutionary model both in male and female models (male: Brownian motion [BM] AIC = 236.27; Ornstein-Uhlenbeck [OU] = 228.56; female: Brownian motion [BM] AIC = 247.67; Ornstein-Uhlenbeck [OU] = 245.78).

Explanatory variables

Prevalence	Lin. richness	Clutch size	Inc. period	Body length	df	AICc	ΔAIC	w_i
Males								
+		+			5	224.01	0.00	0.18
+					4	224.40	0.39	0.15
+		+		+	6	226.45	2.44	0.05
					3	226.45	2.44	0.05
+		+	+		6	226.54	2.53	0.05
+	+	+			6	226.55	2.54	0.05
+				+	5	226.70	2.69	0.04
+	+				5	226.72	2.71	0.04
+			+		5	226.84	2.82	0.04
	+				4	226.96	2.94	0.04
		+			4	227.90	3.89	0.04
Females								
	+		+		5	241.62	0.00	0.17
	+				4	242.30	0.68	0.12
	+			+	5	243.62	2.00	0.06
	+		+	+	6	243.76	2.13	0.06
			+		4	244.06	2.44	0.05
	+	+	+		6	244.08	2.46	0.05
+	+		+		6	244.16	2.54	0.05
+	+				5	244.63	3.01	0.03
					3	244.65	3.03	0.03
	+	+			5	244.70	3.08	0.03
+					4	245.25	3.63	0.02
+			+		5	245.46	3.84	0.02

Table 4: Model-averaged estimates, standard errors, and 95% confidence intervals of variables in the model using the plumage complexity for females and males. Significant variables are marked with asterisks.

Variables	Estimate	Standard Error	95% C.I.
Males			
Intercept	1.49	1.23	-0.96, 3.95
Haemosporidian parasite prevalence	0.64	0.29	0.04, 1.24*
Lineage richness	0.17	0.34	-0.52, 0.87
Clutch size	-0.68	0.43	-1.55, 0.18
Incubation	0.07	0.27	-0.48, 0.62
Body length	-0.09	0.29	-0 68, 0.48
Females			
Intercept	0.23	0.87	-1.52, 1.99
Haemosporidian parasite prevalence	0.19	0.41	-6.63, 1.01
Lineage richness	0.68	0.32	0.02, 1.34*
Clutch size	-0.19	0.50	-1.20, 0.81
Incubation	0.50	0.30	-0.11, 1.12
Body length	0.30	0.37	-0.45, 1.05

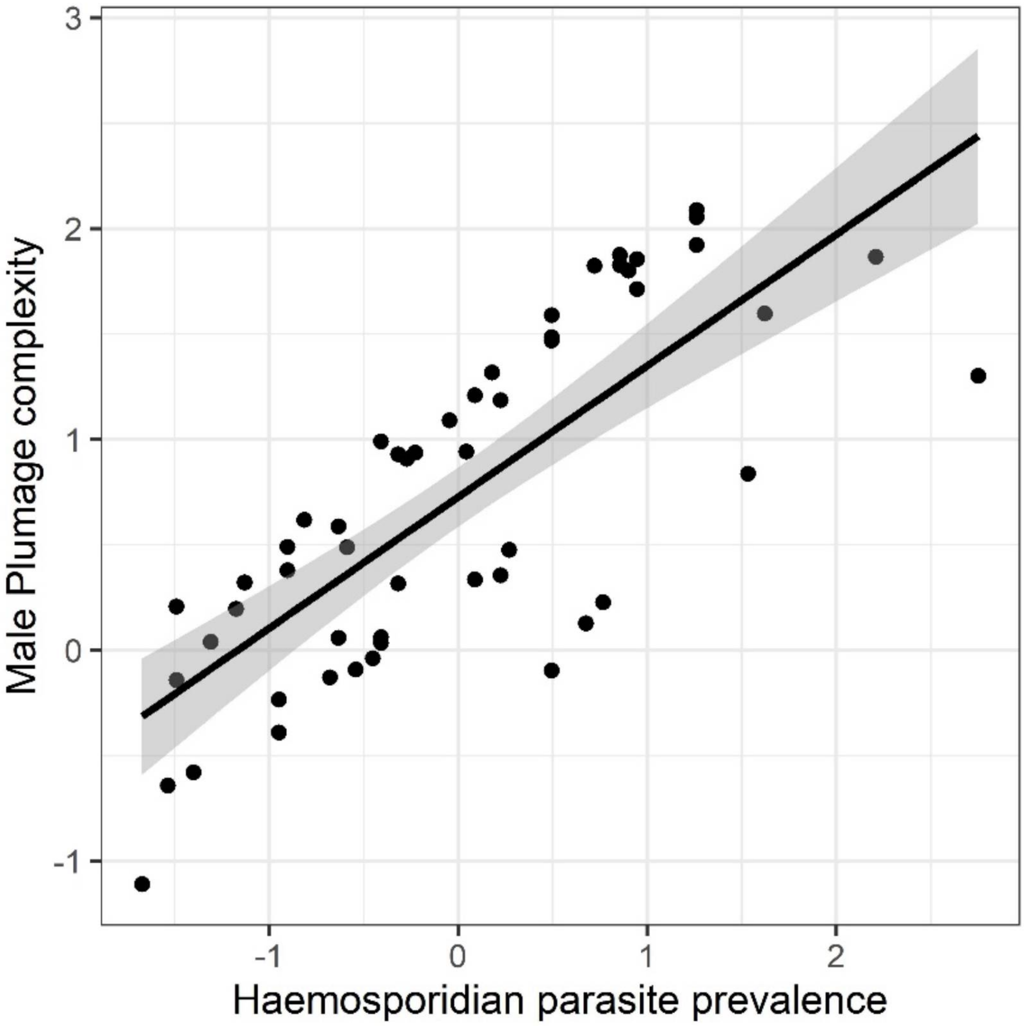

Figure 2: Male plumage coloration complexity in relation to haemosporidian parasite prevalence.

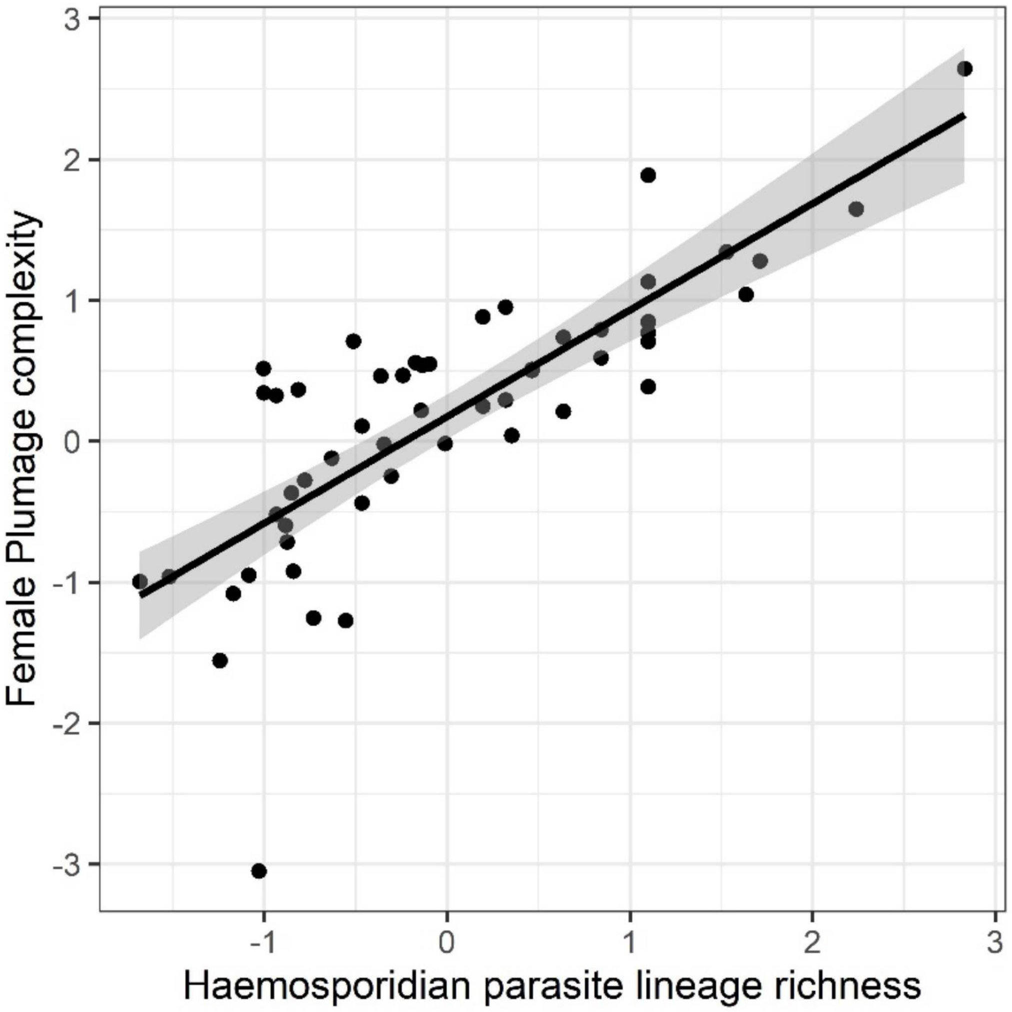

Figure 3: Female plumage coloration complexity in relation to haemosporidian parasite lineage richness.

3.5 DISCUSSION

Here we provide results from one of the broadest studies on haemosporidian parasites in tanager species covering 92 locations throughout the family's geographical distribution. Using parasite prevalence and identified parasite lineages, we build phylogenetic models to test their relationship with plumage dichromatism and coloration complexity. Our main result is suggestive of evidence that the parasite-mediated sexual selection may influence the plumage dichromatism and male and female plumage complexities in tanagers. In brief, more dichromatic species had higher parasite prevalence and were overall smaller in body length. We also found that highly complex male and female plumages were associated with higher haemosporidian parasite prevalence and haemosporidian parasite lineage richness, respectively.

As dichromatism was positively related to higher haemosporidian parasite prevalence, in accordance with our predictions, it is suggestive that host species with a higher proportion of infected individuals appear to be under stronger sexual selection mediated by parasites (Hamilton and Zuk 1982). These results suggest that individuals able to invest in plumage coloration without compromising other physiological functions, such as immunity (Hamilton and Zuk, 1982), are more likely chosen by females for mating, resulting in higher dichromatism in these species. Similar results have been found for rodents (Morand and Bordes 2015) and salamanders (De Lisle and Rowe 2015), suggesting that the relationship between dichromatism and parasites may be widespread in animals. In addition, it was recently shown for 11 non-passerine species that there was an evolutionary correlation between immune and feather pigmentation genes, which gives support for the Hamilton and Zuk (1982) hypothesis (Jaiswal et al. 2021). Also, cell-mediated immunity from better supplemented females may pass onto nestlings, as stated by the transgenerational epigenetics hypothesis (Krüger et al. 2007). Thus, along with Jaiswal et al. (2021) and Krüger et al. (2007), our results indicate that female tanagers select highly conspicuous males as plumage serves as an honest signal for greater immune defenses against haemosporidian parasites, which may have important associations with improved immunity for nestlings. A focus for future studies is the relationship among gene expression, dichromatism, and parasites in tanagers which will help elucidate impacts of sexual selection on genes related to immunity and the overall relationship between immune function and feather pigmentation. Nevertheless, we show that the sexual selection and infection by haemosporidian parasites is associated. Hence, our results could potentially help in identifying disease reservoirs in the wild, since dichromatic species tend to be more parasitized than monochromatic species.

Highly color-complex male and female plumages were associated with higher parasite prevalence and parasite lineage richness, respectively. This result is evidence that parasite-mediated mechanism is also driving the evolution of plumage complexity in both sexes, besides the evolution of dichromatism. Even though sexual selection is expected to be stronger in males, mainly in polygynous breeding systems and in species with males having larger testes (Dunn et al. 2015), our results suggest that female plumage coloration has also evolved under this selection pressure, an evidence which is strengthen by the strong correlation between complexities in male and female colorations (Shultz and Burns 2017). Nonetheless, we should not discard any other social selection process underlying the evolution of female traits, including those within a non-sexual context, such as in territory defense or resource acquisition for increasing fecundity (Tobias et al., 2012). Therefore, our results demonstrate that besides life-history traits, such as foraging stratum, habitat type (species from open habitats being brighter; Shultz and Burns 2017), and male and female plumage coloration evolutionary changes (Shultz and Burns, 2017;

Price and Eaton, 2014), parasitism may have imposed great selective forces on both sexes, which helps understand the evolutionary forces placed upon partially condition-dependent traits in tanagers.

We also found smaller tanagers tend to be more highly dichromatic, which was in accordance with our prediction. Therefore, we suggest that smaller species are under stronger sexual selection, as also reported for parrots (Carballo et al. 2020) and passerine birds in general (Dale et al. 2015). Because individuals of smaller species tend to die younger (short life-span) compared to those of larger species (Ricklefs 2010; Wasser and Sherman 2010), and are under greater risk of predation (Toft and Wright 2015), we suggest that smaller tanager species may have decreased pair bond duration or increased turnover during the breeding season. Therefore, to cope with greater predation threat and sexual selective pressures, secondary traits may aid females in speeding up mate selection and pair bonding, thus reducing the time needed to connect with a prospective and suitable male. Additionally, larger, and longer-lived bird species may have more opportunities for breeding, thus are under weaker selective pressure for breeding. For example, in passerine birds, the sexes of larger species tended to be equally color-complex in their plumages, suggesting that larger individuals may cope better with predation risk, allowing complexity to evolve in both sexes (Dale et al. 2015). Also, larger tanagers produce slower-paced songs and with vocal displays with shorter bandwidth (Mason and Burns, 2015). This result along with ours demonstrate that smaller tanager species are highly dichromatic also have more complex songs and are highly parasitized.

In summary, we found that parasitism by haemosporidian and body size are important traits influencing the plumage dichromatism and coloration complexity in tanager species. Haemosporidian parasite resistance may be an important cue for mate choice and thus parasite-meditated sexual selection (Hamilton and Zuk 1982) should favor plumage dichromatism in these species. Also, smaller tanager species tended to be more dichromatic, possibly because of shorter pair bonds or increased individual turnover during the reproductive period.

Tanagers with their diversity of life-history traits, and coloration allow for the understanding the complex interplay between sexually selected traits and parasitism. Our results demonstrate that within such a diverse group, sexual selection has produced higher trait variability in those species under higher risk of infection. Additional work is warranted to determine whether these traits serve as honest signals of immune response to parasitism.

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4.0 Capítulo 3: Relationship between feather corticosterone, haemosporidian parasite occurrence and plumage coloration in two bird species

Relationship between feather corticosterone, haemosporidian parasite occurrence and plumage coloration in two bird species

4.1 ABSTRACT

The body condition is a multi-level characteristic related to several physiological metrics, such as immunity, corticosterone, and plumage coloration in birds. However, there may be multiple directionalities of cause and effect such that condition affects physiology or physiology affects condition. Therefore, our objective here was to study the relationship among several physiological variables, namely: plumage coloration (hue and saturation), haemosporidian parasite occurrence (presence x absence), feather corticosterone presence and body condition in the white-browed warbler and the rufous gnateater. We captured birds in a Brazilian natural reserve, in Piraquara, Paraná State, Brazil. We screened 22 white-browed warblers and 12 rufous gnateaters for haemosporidian infection and measured the concentration of feather corticosterone through an ELISA, to procude standard curves of corticosterone concentrations from absorbance values. We performed structural equation modeling that allowed us to test multiple regressions and covariance models. We found a positive association between corticosterone deposited in feathers and haemosporidian parasite occurrence in white-browed warblers, suggesting that either the prolonged exposure to corticosterone suppressed the immunity and made individuals more susceptible to parasitism, or that parasitism increased corticosterone levels in these birds. Finally, we found a negative association between plumage color saturation and haemosporidioses in rufous gnateaters, suggesting that infected individuals were less capable of acquiring carotenoid-rich resources or that they invested in immunity at the expense of plumage coloration. Our results show that haemosporidian parasite infection is related to stress and plumage color in two species from southern Brazil.

Keywords: color hue, saturation, carotenoid chroma, ELISA, *Plasmodium*, *Parahaemoproteus*, *Leucocytozoon*, scale-mass index.

4.2 INTRODUCTION

Body condition is a multi-level characteristic (also mentioned as health status, health condition, or simply condition in the literature), that relates the nutritional status of organisms with immunity, energy reserve, and foraging ability (Brown, 1996). Indices such as the residuals of a regression between mass-body measurement (usually the tarsus) (Peig and Green, 2009, 2010; McGraw *et al.*, 2020) are frequently used to represent individual condition in bird studies. This residual index explains 50% of the fat content, as demonstrated in a multi-level study, involving more than 200 species (Labocha and Hayes, 2012). Consequently, there is more than just the relationship between the mass and a body measurement that may modulate individual fitness, such as the habitat patch interconnectedness. Energy expenditure influences the body condition such that birds inhabiting more interconnected forested patches should be in better condition that those from more sparse patches (Molina-Marin *et al.*, 2022). On the other hand, the body condition can be influenced by the resource availability as well as the individuals' ability to acquire energy-rich resources. For example, individuals of blue petrels (*Halobaena caerulea*) and American dippers (*Cinclus mexicanus*) that could find energy-rich resources more frequently, and therefore with higher foraging efficiency, had an overall better body condition (Donnelly and Sullivan, 1998, Weimerskirch *et al.*, 2003). Also, body condition of swallowed-tailed manakins from southeast Brazil have a decreasing pattern over time, potentially due to increased droughts, habitat loss and the expansion of the agricultural land use (Penha and Rodrigues, 2022), suggesting the key role that stress play in the condition physiology.

Stress, which is any change to the homeostasis (Romero, 2012), is a hormone-regulated physiological condition. In birds, the most common stress hormone is the corticosterone (CORT), controlled by the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky *et al.*, 2000). CORT secretion changes the behavior and increases survival in stressed organisms (Bortolotti *et al.*, 2008). However, prolonged exposure to stressful sources and high CORT levels in the bloodstream may have detrimental impacts on individuals, such as decreased TLR-5 gene expression, reduced innate immunity in broilers (Yang *et al.*, 2015) and lower ability to cope with haemosporidioses (Names *et al.*, 2021). Haemosporidian parasites (Order Haemosporidae; genera *Plasmodium*, *Parahaemoproteus* and *Leucocytozoon*) are vector-borne protists and cause a malaria-like disease that occurs worldwide in several different bird families (Valkiunas, 2005). Malarial parasites, even in the chronic state of infection, may cause negative effects on individuals and impact body condition. For example, a lower body mass/tarsus relationship was predicted by a higher parasitemia (proportion of infected red-blood cells) in different bird species from the Brazilian Cerrado (da Silva Rodrigues *et al.*, 2021). The authors hypothesized that individuals with a worse body condition had a higher susceptibility to more severe cases of haemosporidioses. In contrast, in the same biome, higher haemosporidian parasite prevalence (proportion of infected individuals) were linked to a higher body condition, suggesting that better-fit individuals may clear-off malarial parasite more easily than less-fit ones (Ribeiro *et al.*, 2020). These contrasting results indicate that more physiological variables are involved in the relationship between stress and body condition, such as secondary sexual traits.

Birds are highly visual organisms (Hill and McGraw, 2006) and the plumage coloration is used as an honest condition-dependent trait to mate choice (Andersson, 1999). The coloration mainly comes from carotenoids, melanin, and structural coloration; the first can only be acquired through the diet, the second is a result of the protein metabolism, and the last is mainly related to the cellular structure of feathers, making light have differential reflectance (Hill and McGraw, 2006). Carotenoids for instance are used as immune stimulators such that individuals that are able to find carotenoid-rich resources may be better-equipped to fight-off parasites (Saks *et al.*, 2003; Giraudeau *et al.*, 2013; McGraw *et al.*, 2013). Carotenoids are also linked with the body condition, suggesting that birds in a better condition have more conspicuous coloration. For example, in males of red fody (*Foudia madagascariensis*) with a brighter breast plumage coloration were in a better health status (Hayes *et al.*, 2006). Also, less colorful plumages in carotenoid-containing patches were associated with individuals being more likely infected by multiple genera of haemosporidian parasites in blue tits (del Cerro *et al.*, 2010), or having a worse body condition in black-legged kittiwakes (Leclaire *et al.*, 2019). Melanin may also have an important link with immunity, and studies involving different morphs of feral pigeons have shown that. Therefore, darker morphs with higher melanin levels have higher cellular immune response and are less likely infected by haemosporidians compared to lighter morphs (Jacquin *et al.*, 2011; but see Aouissi *et al.*, 2021). In addition, melanin-based plumage coloration may also indicate a better condition. For example, females of Eurasian kestrels (*Falco tinnunculus*) with higher chroma on the dark rump were in a better condition and mounted a higher innate immunity (Parejo *et al.*, 2011).

Therefore, there is an overlapping relationship between bird body condition, plumage coloration, stress (CORT concentration) and haemosporidioses, since they may all be related to the health status of an organism. Here we tested the relationship between these variables in two species, the rufous gnateater (*Conopophaga lineata*, Wied-Neuwied, 1831, Passeriformes: Conopophagidae) and the white-browed warbler (*Myiothlypis leucoblephara*, Vieillot, 1817, Passeriformes: Parulidae) in a south Brazilian Atlantic Forest reserve. We used structural equation modeling (SEM) to consider the relationship among multiple variables (Wright, 1921) since stress and immunological responses, the maintenance of body condition and production of plumage coloration may share mutual physiological mechanisms. Consequently, we did not have specific expectations based on our variables, since there were multiple possibilities. Therefore, we proposed several hypotheses based on previous studies that can be found in the supplementary table 1.

4.3 METHODS

4.3.1 Study species

The rufous gnateater is slightly sexual dimorphic, with males and females having a rufous-chestnut crown with a grey supercilium. However, males have a silvery/whitish extension of the supercilium (Whitney *et al.*, 2020). The rufous gnateater occurs in southeastern and southern Brazil, southeastern Paraguay, and a small portion of the Northeastern part of Argentina. On both sexes, there is a whitish patch on the belly, and a rufous chin, throat, and breast (Whitney *et al.*, 2020). The white-browed warbler occurs in southeastern South America, namely in Argentina, Brazil, Paraguay, and Uruguay (Curson and Kirwan, 2020). Sexual dimorphism is not apparent in this species, and both males and females have a gray head with black/gray lateral crown and an eye stripe. The throat is whitish, the breast is grayish, also presenting an olive-green coloration on the flanks and orange legs (Curson and Kirwan, 2020).

4.3.2 Study site and data collection

The study was performed at the Pico do Marumbí State Park (48° 59’ W 25° 29’ S), located in Piraquara municipality, state of Paraná, Brazil. The reserve is part of the Brazilian Atlantic Forest biome, in the western region of the Serra do Mar Mountain, with a typical rainforest (Reginato and Goldenberg, 2007). Individuals were captured using ten mist nets with 36 mm mesh, 12 meters long and 2.5 meters high. The nets were opened ten minutes before sunrise, and closed six hours afterwards, from June to September 2019 (Licenses approval numbers: Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio 68579-1; Instituto Ambiental do Paraná – 15.19; Animal Use Ethics Certificate of the Universidade Federal do Paraná – 23075.027928/2019-81). For each captured individual, we collected the body mass using a digital scale to the nearest 0.1 g, and right-wing length with a caliper to the nearest 0.01 mm, and both measurements were used to estimate the body condition (see below). All individuals were banded according to the standards established by the National Center for Research and Conservation of Wild Birds (CEMAVE/IBAMA – License 4925930). A blood samples of up to 50 µL were collected through a puncture in the brachial vein of the left wing. The blood sample was stored in a labelled microtube with absolute ethanol for haemosporidioses analysis. To measure the plumage coloration (see below), we also collected 5-6 feathers from a single patch in each species: (a) rufous gnateater: the rufous breast; (b) white-browed warbler: the grayish patch on the breast. We kept all feathers on labeled microtubes protected with aluminum foil until analysis. Finally, we collected the nineth and tenth primary flight feather from the left wing to measure corticosterone (see below). After data collection, individuals were released in the same place they were captured.

4.3.3 Body condition index

We measured the body condition for each species separately, as the residuals of a mass-wing length regression (e.g. Peig and Green, 2009; Dias *et al.*, 2016; Colorado and Rodewald, 2017; Nip *et al.*, 2018; De La Torre *et al.*, 2020; McGraw *et al.*, 2020; Penha *et al.*, 2020; Ribeiro *et al.*, 2020; da Silva Rodrigues *et al.*, 2021; Molina-Marin *et al.*, 2022; de Souza Penha and da Silva Rodrigues, 2022). We found a significant regression between the mass and the wing length for both species (white-browed warbler: $F_{1,22} = 47.31$; $p < 0.01$; $R^2 = 0.68$; rufous gnateater: $F_{1,12} = 61.27$; $p < 0.01$; $R^2 = 0.84$). Residuals indices may have limitations compared to other metrics, mainly when comparing birds from different populations (Labocha and Hayes, 2012). However, we consider it adequate for our purpose since we are comparing individuals of the same species from the same location (Andersson *et al.*, 2002; Giraudeau *et al.*, 2013; Wojczulanis-Jakubas *et al.*, 2015; Salleh Hudin *et al.*, 2016; Nip *et al.*, 2018; da Silva Rodrigues *et al.*, 2021).

4.3.4 Haemosporidian parasite analysis

To identify haemosporidian parasite occurrence, we used a multiplex polymerase chain reaction (Multiplex PCR). Following the protocol described by Ciloglu *et al.* (2020). Therefore, we used the haemosporidian parasite occurrence as our variable of parasitism, with or without recorded infection. Descriptively, we also calculated the prevalence of haemosporidian parasites for each species, as the number of infected individuals divided by the total captures.

4.3.5 Feather corticosterone analysis

Since we are studying the relationship between plumage coloration, and a possible chronic infection of haemosporidian parasites, we analyzed the corticosterone in feathers, which is thought to be a long-term stress indicator (Bortolotti et al., 2008). Therefore, before analysis, feathers were weighted to the nearest 0.0001g using a digital scale (BEL

- M214Ai). Then, the calamus was isolated from every feather and cut into small pieces (<5 millimeters) to extract the corticosterone. 10 mL of methanol was added to the calamus pieces and left in a water bath with sonication at room temperature for 30 minutes. The samples were then incubated at 50°C for 12 hours in a water bath with running water. The methanol was separated by vacuum filtration and then twice washed with 2.5 mL of methanol and let air-dried (miVac duo concentration, Genevac™). Phosphate buffer were then added to the filtrated samples and frozen at -20°C until analysis. Finally, we performed an ELISA assay using the Corticosterone Competitive ELISA Kit (Invitrogen). The ELISA was performed according to the manufacturer's instructions. A relative standard curve was constructed from the absorbance values according to the control available in the commercial kit. We estimated corticosterone concentration using data interpolation with Pade (1,1) approximant (Graph pad prism 9.1). The final CORT concentration was in function of the weight of the feather (pg/ug).

4.3.6 Plumage coloration analysis

We placed the feathers from each patch separately on a previously tested black card without ultraviolet reflection. Using a portable spectrophotometer (USB4000; Ocean Optics) and the program Spectrasuite 12.2 (Ocean Optics), we positioned the feathers overlapped on the dark background and measured their reflectance. We considered the reflectance wavelengths between 300 and 700 nanometers, and then used the PAVO package (Maia *et al.*, 2013) from the R software (R Core Team, 2019) to extract the saturation and hue considering the bird tetrahedral color space visual system (Vorobyev and Osorio, 1998; Stoddard and Prum, 2008). We used the achieved saturation ("saturation" hereafter), i.e. the ratio between the distance from the achromatic center and the maximum saturation (Maia *et al.*, 2013), whereas the hue was divided into two angles, theta and phi. The angles represent the longitude and latitude of points within the visual system of birds in the tetrahedron (Vorobyev and Osorio, 1998). Since both values of hue were correlated for both species (54% for the white-browed warbler and 45% for the rufous gnateater), we only kept the theta in our analysis ("hue" hereafter). The saturation of the rufous gnateater had four discrepant values (discrepant values being 40% higher, and the rest closer to zero), so we categorized this variable in low (below the median of the saturation) and high levels (above the median of the saturation; histogram can be found in supplemental figure 3).

4.3.7 Statistical analysis

We first visually inspected the distribution of numeric variables. We found that the feather corticosterone had six high values for the white-browed warbler and three for the rufous gnateater (discrepant values were higher than 1000 pg/ug, and the remaining values were closer to zero or non-detectable). We then categorized this variable into two levels: low (feather corticosterone lower than 100 pg/ug) and high (feather corticosterone higher than 1000 pg/ug) for both species. For the remainder variables, we used logarithmic or square root transformations to normalize their distribution whenever it was necessary. We standardized all variables using the *scale* function from R software v. 4.2.1 (R Core Team, 2022). We then performed two structural equation modeling (SEM), one for the white-browed warbler and another for the rufous gnateater. The models and predictions for each species can be found in supplementary table 1. SEM is an important mathematical approach to determine the relationship among several metrics (Grace *et al.*, 2012) tested through regressions and covariances. In regressions, we hypothesized the relationships based on previous studies (see supplementary table 1), considering the plumage coloration saturation and hue as response terms and body condition, haemosporidian parasite occurrence and CORT concentration as predictor variables. Whenever we had evidence for a mutual relationship, we generated covariances. For example, body condition has already been shown to influence the occurrence of haemosporidian parasites (da Silva Rodrigues *et al.*, 2021), but the occurrence of haemosporidian parasites may also influence body condition (Ribeiro *et al.*, 2020). Covariances were denoted with a double tilde. We used the *sem* function from the *lavaan v. 4.2* (Rosseel, 2012) package to perform the modeling and visually inspected the normality of statistical significant residuals using a scatter plot. All significant models had normally distributed residuals. We used the following metric as goodness of fit test (cut-off values in parenthesis): root mean square error (RMSEA – lower than 0.06) (Fan *et al.*, 1999); the root mean square residual (SRMR – values close to zero) (Feinian Chen *et al.*, 2008) and the Tucker-Lewis index (TLI – higher than 0.90) (Hu and Bentler, 1999). We considered the path as significant if the p-value was lower than 0.05. All analyses were performed in the R software (R Core Team, 2019).

4.4 RESULTS

We found an apparent higher prevalence (proportion of infected individuals) with haemosporidian parasites in rufous gnateater than in white-browed warblers, whereas more individuals of white-browed warblers had a high concentration of corticosterone in the feathers compared to the individuals of rufous gnateater (Table 1).

Table 1: Summary data for the white-browed warbler and the rufus gnateater indicating the number of captures (n), haemosporidian parasite prevalence (proportion of infected individuals – P), average wing length (in cm, W), mass (in g, M), saturation (Sat), hue theta, feather CORT concentration (proportion of individuals with high against low CORT concentration), and sex (proportion of males to females).

Species	n	Sex	P	W	M	Sat	Theta	CORT
White-browed warbler	22	NA	18%	13.98 ± 3.00	16.09 ± 0.31	1.37 ± 2.44	-1.13 ± 1.19	37% (6:16)
Rufous gnateater	12	3 M: 9 F	33%	19.2 ± 3.90	24.02 ± 1.79	4.54 ± 14.23	-0.59 ± 0.39	33% (3:9)

We found that the SEM for white-browed warblers was a good model (Supplementary Table 2) and indicated a significant association between CORT and haemosporidian parasite occurrence (Table 2). Therefore, individuals with high CORT concentration deposited in the feather were also more likely parasitized by haemosporidian parasites (Table 3, Figure 1).

Table 2: Structural equation modeling with the regressions and covariance results for the white-browed warbler.

Model	**Estimate**	**se**	**z-value**	**p-value**
Regressions				
Saturation ~				
Corticosterone +	-0.03	0.44	-0.08	0.93
Haemosporidian parasite occurrence +	-0.83	0.54	-1.53	0.12
Body condition	-0.17	0.11	-1.47	0.14
Hue ~				
Corticosterone +	0.75	0.63	1.19	0.23
Haemosporidian parasite occurrence +	-0.29	0.76	-0.37	0.70
Body condition	-0.01	0.16	-0.06	0.95
Covariances				
Hue ~~ Saturation	-0.20	0.20	-1.04	0.29
Corticosterone ~~ Haemosporidian parasite occurrence	0.08	0.04	2.11	0.03*
Body condition ~~ Corticosterone	-0.06	0.14	-0.44	0.65
Body condition~~ Haemosporidian parasite occurrence	-0.19	0.13	-1.44	0.15

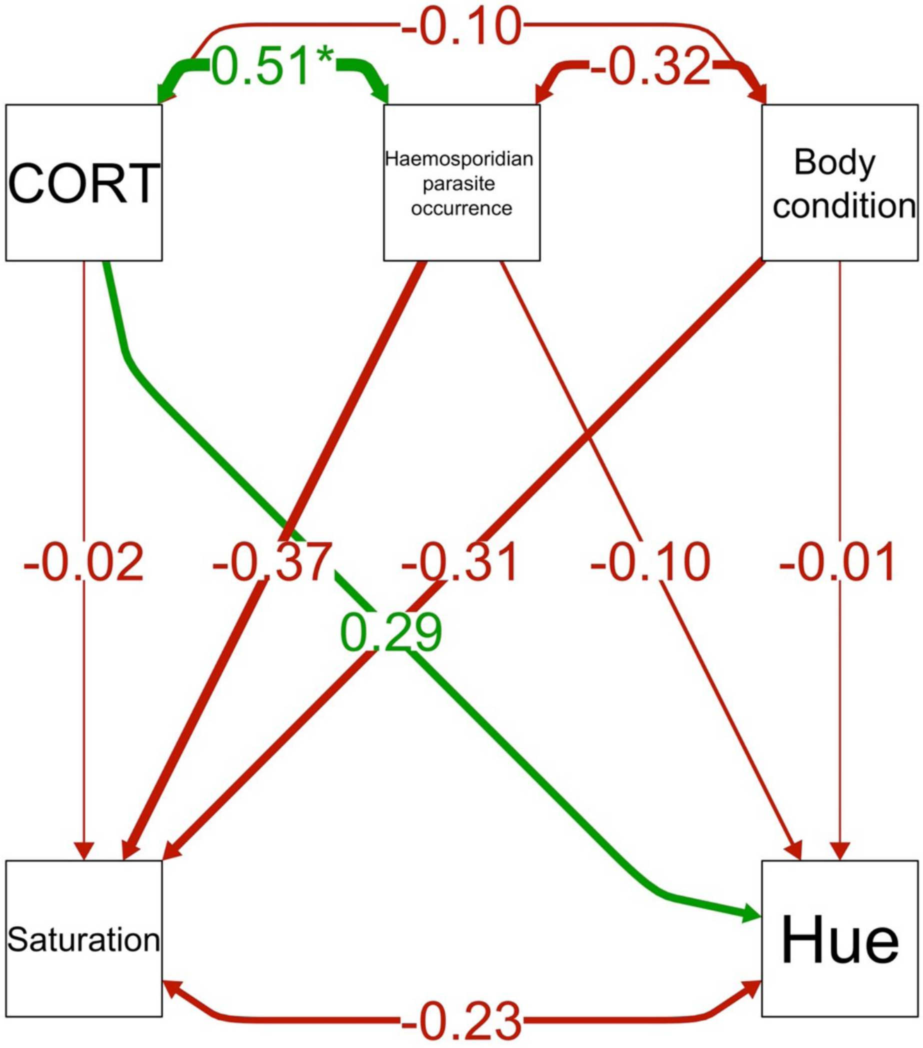

Figure 1: Structural equation modeling diagram for the white-browed warbler, with boxes showing the variables (CORT: corticosterone concentration; haemosporidian parasite occurrence (presence x absence of infection); body condition; saturation and hue). Values indicate the standardized parameter estimates. Red and green line colors indicate a negative and positive relationship, respectively, whereas the width of lines indicates strength of the association among variables, being thicker lines more strongly associated. Statistically significant relationships are shown with an asterisk.

Similarly, , we observed a significant association between plumage coloration saturation and haemosporidian parasite occurrence for rufous gnateater (Supplementary table 2) (Table 4). Therefore, infected individuals with haemosporidian parasites had a lower feather color saturation (Table 4, Figure 2).

Table 4: Structural equation modeling with the regressions and covariance results for the for the rufous gnateater.

Model	**Estimate**	**se**	**z-value**	**p-value**
Regressions				
Saturation ~				
Corticosterone	0.16	0.27	0.59	0.55
Haemosporidian parasite occurrence	-0.75	0.28	-2.64	0.00*
Body condition	-0.02	0.08	-0.30	0.76
Hue ~				
Corticosterone	0.12	0.18	0.70	0.48
Haemosporidian parasite occurrence	-0.03	0.19	-0.16	0.87
Body condition	-0.02	0.05	-0.35	0.72
Covariances				
Hue ~~ Saturation	0.00	0.02	0.08	0.93
Corticosterone ~~ Haemosporidian parasite occurrence	0.08	0.06	1.30	0.19
Body condition ~~ Corticosterone	0.83	0.24	1.58	0.11
Body condition ~~ Haemosporidian parasite occurrence	0.53	0.27	1.90	0.05

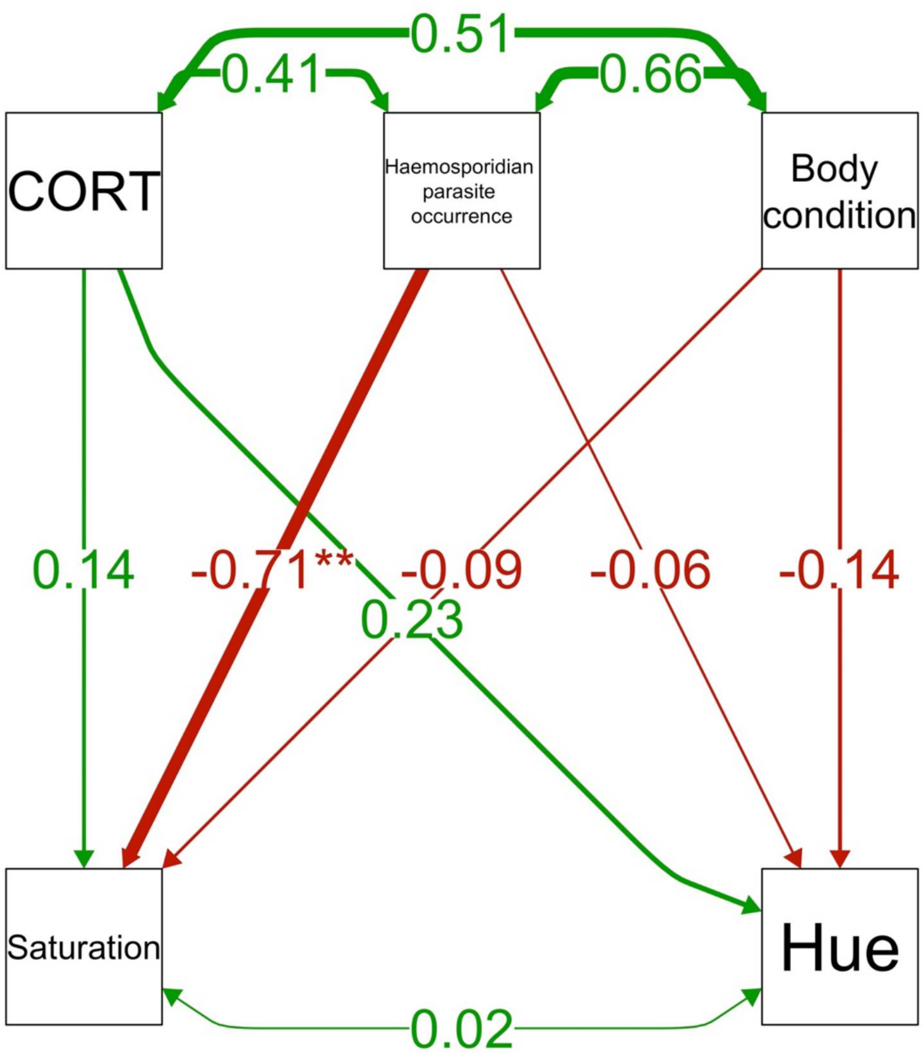

Figure 2: Structural equation modeling diagram for rufous gnateater, with boxes showing the variables (CORT: Corticosterone concentration; haemosporidian parasite occurrence; body condition; Saturation and Hue and sex). Values indicate the standardized parameter estimates. Red and green line colors indicate a negative and positive relationship, respectively, whereas the width of lines indicates strength of the association among variables, being thicker lines more strongly associated. Statistically significant relationships are shown with an asterisk.

4.5 DISCUSSION

We found that the occurrence of haemosporidioses covaried positively with high CORT deposited in the feathers for the white-browed warbler. Additionally, we also found a negative association between haemosporidian parasite occurrence and plumage coloration saturation in the rufous gnateater, meaning that individuals with a low saturation were more commonly infected with haemosporidian parasites. Body condition and hue were associated with any other variables in our models.

The positive covariance between the presence of corticosterone deposited in the flight feathers of white-browed warblers and the occurrence of haemosporidian parasites is evidence that infection by malarial parasites is linked to stress. Corticosterone is the main hormone produced during adverse contexts, such as those imposed under high predation risk or food deprivation (Bortolotti *et al.*, 2008). Also, when produced for a prolonged time, circulating levels of CORT tend to decrease the inflammatory response and the likelihood of fighting-off parasites (Cornelius *et al.*, 2014). Therefore, since we do not know the exact instance of infection by haemosporidians, our results support two non-exclusive hypotheses: (a) immunosuppressed individuals with higher CORT levels were more susceptible to infection; or (b) infected individuals with haemosporidian parasites produced and deposited more CORT in the feathers in response to the parasitism. Results for the first hypothesis have been controversial, with studies demonstrating a lack of (Bichet *et al.*, 2020 - in house sparrows [*Passer domesticus*, Passeriformes: Passeridae]; Bosholn *et al.*, 2020 - in blue-crowned manakins [*Lepidothrix coronata*, Passeriformes: Pipridae]) or a positive association (Names *et al.*, 2021 *Chlorodrepanis virens*, Passeriformes: Fringillidae). Studies supporting the second hypothesis have been shown in house finches (*Haemorhous mexicanus*, Passeriformes: Fringillidae) infected by *Mycoplasma gallisepticum*, which causes a conjunctivitis-like disease in birds (Love *et al.*, 2016). Nevertheless, our results show an important relationship between corticosterone and haemosporidioses occurrence in a free-ranging population of white-browed warblers.

We also found a negative relationship between plumage color saturation and haemosporidioses in rufous gnateaters. The achieved saturation is an indicative of the color purity, and may be related to the ability of acquiring carotenoid-rich resources (Hill and McGraw, 2006; Senar *et al.*, 2008), since higher saturation is linked to a higher carotenoid deposition in feathers (Saks *et al.*, 2003). Therefore, our results suggest again two non-mutually exclusive hypothesis: (a) infected individuals with haemosporidian parasites were less likely to compete successfully and acquire carotenoid-rich resources, resulting in a less saturated plumage coloration; or (b) infected individuals with malarial parasites traded the investment in feather carotenoid deposition in feathers to the immune system to fight-off parasites. Evidence for the first hypothesis have been shown in an experimental study in blackbirds (*Turdus merula,* Passeriformes: Turdidae). Carotenoid-supplemented birds with brighter bill coloration decreased the replication of a gut parasite (*Isospora* sp.), suggesting that birds that are better equipped to finding resources may be more able to fight off parasites and have a brighter coloration (Baeta *et al.*, 2008). On the other hand, support for the second hypotheses rely on studies showing that the plumage coloration was predicted by the plumage coloration in birds (Figuerola *et al.*, 1999). For example, in lesser elaenias, flavescent warblers and red pileated finches, infected individuals with haemosporidian parasites had a lower carotenoid chroma deposited in the feathers compared to uninfected individuals (Penha *et al.*, 2020). The authors hypothesize that infected individuals may have less carotenoid available for feather pigmentation and may invest more in immunity stimulation to fight-off parasites, which supports our second hypothesis.

We did not find the body condition as an important variable relating to any other physiological metric. The mass/wing regression may be considered as an honest health and nutritional status in birds (Brown, 1996). Although body condition has been linked to the habitat connectedness (Molina-Marin *et al.*, 2022), resource availability, foraging efficiency (Donnelly and Sullivan, 1998; Weimerskirch *et al.*, 2003), haemosporidian parasite parasitemia and prevalence (Ribeiro *et al.*, 2020; da Silva Rodrigues *et al.*, 2021), carotenoid-containing plumage brightness (Hayes *et al.*, 2006; Leclaire *et al.*, 2019), and melanin-containing plumage coloration (Parejo *et al.*, 2011), we did not find support for that. In addition, our results suggest that the parasitism by haemosporidian parasites may not directly impair the condition in individuals. Therefore, it is possible that the majority of the individuals captured in our study were in a chronic phase of infection, with little effect to the overall condition in individuals, which has been demonstrated elsewhere (van de Crommenacker *et al.*, 2012; Granthon and Williams, 2017). Also, plumage hue and saturation did not relate to body condition, which was similar in lesser goldfinches (*Spinus psaltria*). This result may suggest that the rufous patch of rufous gnateaters and the grayish patch of white-browed warbler may relate to other physiological variables, such as the clutch size (Morrison *et al.*, 2014). In addition, the body condition did not relate to the feather CORT in our study, which was similar in artic-nesting migratory bird species (Legagneux *et al.*, 2013). Therefore, feather corticosterone, considered as a reliable measure of long-term stress, may relate to parasitism, but may have little effect to the overall condition of birds. We believe that due to our low sample size some of the relationships may have been compromised, even though our models showed robust results. However, more studies are needed with larger samples and with more species to better understand the multiple relationships among our studied variables.

In summary, we demonstrated an important association between a stress hormone deposited in feathers, the corticosterone, and the occurrence of haemosporidioses in white-browed warblers. Also, infected rufous gnateaters had a less saturated plumage coloration. However, support for any relationship between body condition and any other physiological variable was not found. Therefore, in a study involving several condition-dependent traits, such as the body condition, plumage coloration, stress hormone and haemosporidian parasite occurrence, we found that the parasitism is an important factor associated with immunity and secondary traits, which may interfere in mate selection processes.

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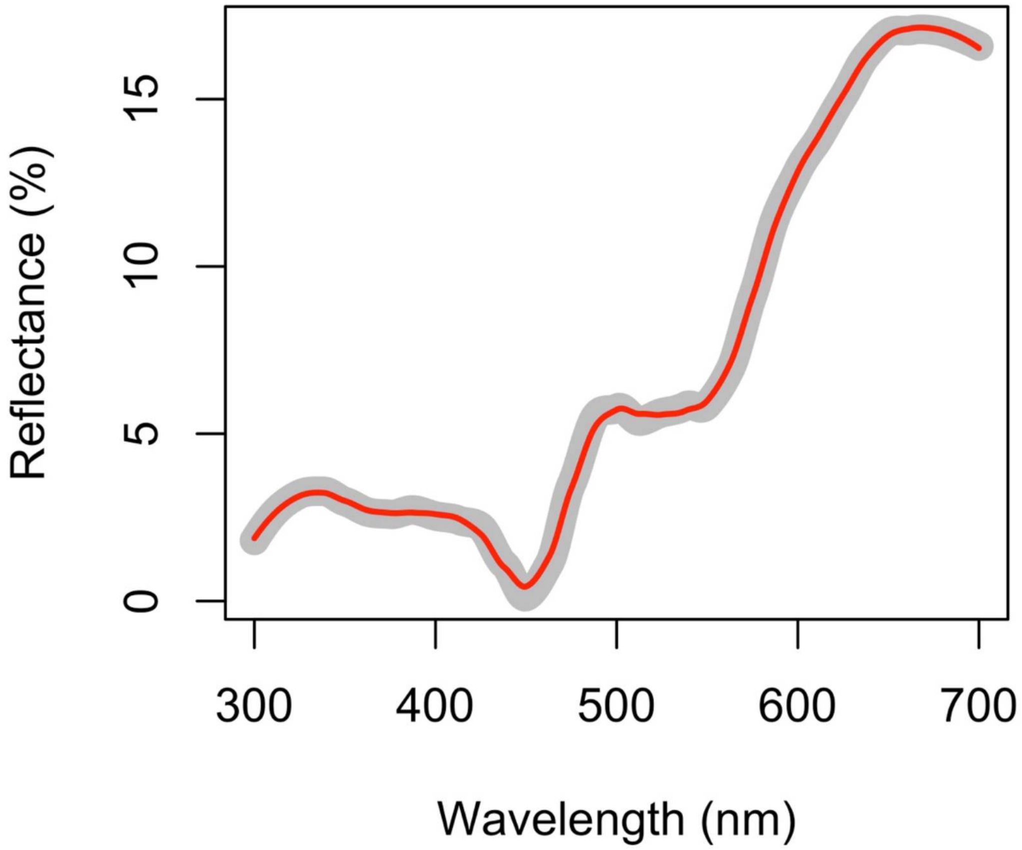
4.7 SUPPLEMENTARY MATERIAL

Table 1: Models and predictions of the two structural equation modeling for the white-browed warbler and the rufous gnateater.

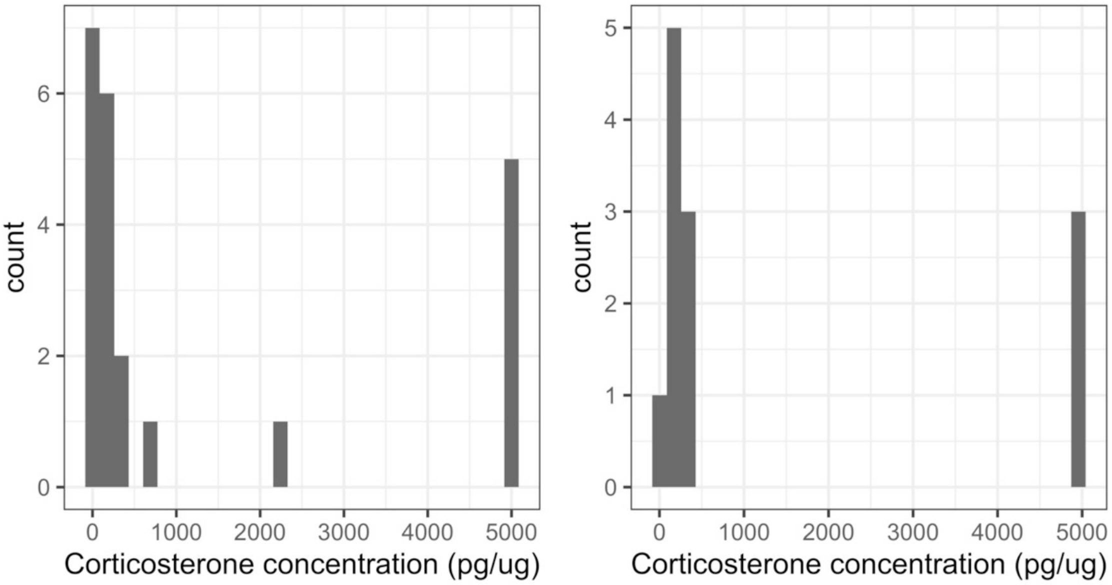
Regression models	**Prediction**
Saturation ~ Corticosterone + Haemosporidian parasite + body condition	Negative association between saturation / hue and both haemosporidioses and feather corticosterone level (Sundberg, 1995; Vleck *et al.*, 2000; Saks *et al.*, 2003b; Hill *et al.*, 2004; Lattin *et al.*, 2011; Rotenberry *et al.*, 2014; Romano *et al.*, 2019; Penha *et al.*, 2020b). Positive association between saturation / hue and body condition (Spears and Cavitt, 2003; Dias *et al.*, 2016; Granthon *et al.*, 2017; De La Torre *et al.*, 2020; Penha *et al.*, 2020b).
Hue ~ Corticosterone + Haemosporidian parasite + body condition	Negative association between hue and both haemosporidioses and feather corticosterone level (Sundberg, 1995; Vleck *et al.*, 2000; Saks *et al.*, 2003b; Hill *et al.*, 2004; Lattin *et al.*, 2011; Rotenberry *et al.*, 2014; Romano *et al.*, 2019; Penha *et al.*, 2020b). Positive association between hue and body condition (Spears and Cavitt, 2003; Dias *et al.*, 2016; Granthon *et al.*, 2017; De La Torre *et al.*, 2020; Penha *et al.*, 2020b).
Covariances	
Hue ~~ Saturation	Positive relationship between hue and saturation (Hill and McGraw, 2006).
Corticosterone ~~ Haemosporidioses	Positive relationship between corticosterone levels in the feather and haemosporidioses (Names *et al.*, 2021a).
Body condition ~~ Haemosporidioses	Negative relationship between body condition and haemosporidioses (Ribeiro *et al.*, 2020a; da Silva Rodrigues *et al.*, 2021a).
Body condition ~~ Corticosterone	Negative relationship between corticosterone levels and body condition (Heath and Dufty, 1998).

Supplementary table 2: Structural equation modeling results, indicating the Tucker-Lewis' index (TLI), the root means square error of approximation (RMSEA) and the standardize root mean square residuals for both species, namely the white-browed warbler and the rufous gnateater.

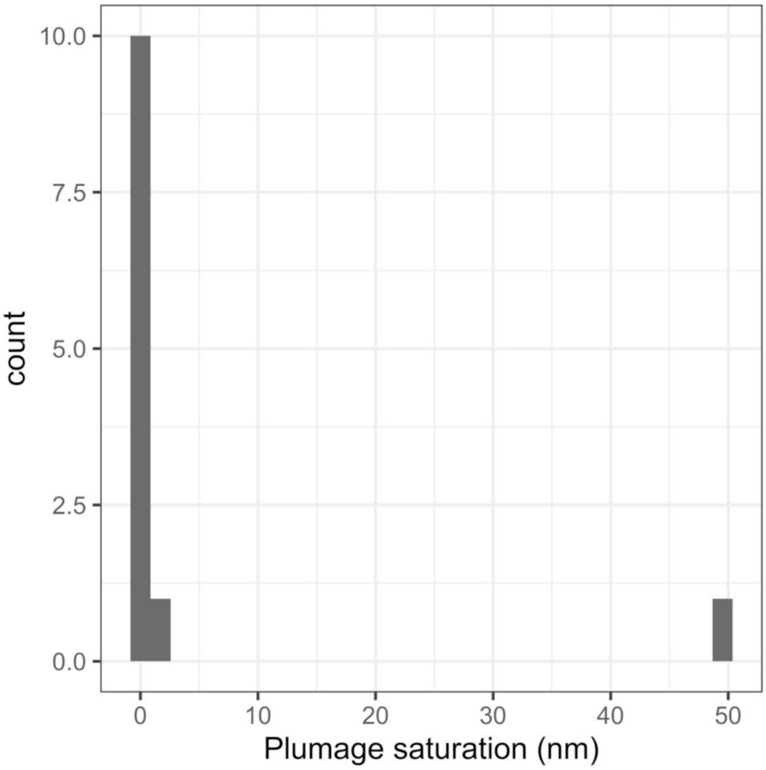
Species	**TLI**	**RMSEA**	**SRME**
White-browed warbler	1.000	0.000	0.000
Rufous gnateater	1.000	0.000	0.000



Supplemental figure 1: Relationship between the reflectance of feathers from the rufous gnateater (*Conopophaga lineata*) and wavelengths (nm). Curve shows a reddish to orangish coloration.



Supplemental figure 2: Histogram of the concentration of corticosterone from the flight feathers in white-browed warbler (left) and rufous gnateater (left).



Supplemental figure 3: Histogram of the plumage saturation in rufous gnateaters.

5.0 Capítulo 4: Infection with coccidia and malaria in house finches, and their relationships to several metrics of condition, and plumage coloration

Manuscript to be submitted for publication to International Journal of Parasitology or Journal of Wildlife Diseases

Infection with coccidia and malaria in house finches, and their relationships to several metrics of condition, and plumage coloration

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5.1 ABSTRACT

Pathogen and parasite species traditionally have been studied as single infectious systems with their hosts, but in nature they rarely infect hosts in isolation and often co-occur. In such situations, we still have much to learn about co-infection dynamics – including both occurrence and severity of the diseases – as well as how the life-history traits of host species relate to likelihoods and degrees of co-infection or single infection. We examined infection occurrence and severity of two parasites – a gut parasite (*Isospora* coccidians) and a blood parasite (*Plasmodium* sp) – in house finches (*Haemorhous mexicanus*), a common and widespread passerine bird in North America, and related parasitic infections to several health- and condition-related metrics in the birds (blood glucose concentration, body condition, total leukocyte count, heterophil/lymphocyte ratio, and circulating levels of carotenoids and vitamins E). We captured 55 birds (22 males, 33 females) in October 2021 in Tempe, Arizona, USA and used blood and fecal samples to screen individuals for malarial and coccidian parasites, respectively. We found no significant relationships between the presence or severity of the two parasites in individual house finches. However, the presence of malarial parasites was significantly predicted by higher heterophil/lymphocyte ratio and circulating levels of the carotenoid lutein. Also, birds with high levels of malaria parasitemia had a high heterophil/lymphocyte ratio, suggestive of elevated stress levels from infection. Coccidiosis severity was negatively predicted by circulating vitamin E levels. Finally, male plumage coloration was best predicted by 3’-hydroxy-echinenone, indicating that redder birds had higher levels of this circulating carotenoid. Our study sheds light on the complex relationships involving multiple parasites in a wild bird system and on how a series of hematological-, condition-, and health-related parameters can variably relate to different types of parasitic infections.

Keywords: Haemosporidian parasites, coccidiosis, circulating carotenoids, global leucocytes, glucose concentration, heterophil/lymphocyte ratio, malarial parasites.

5.2 INTRODUCTION

Wild animals are affected by many different parasites and pathogens during their lifespan (Wobeser 2009). Therefore, studying natural patterns of disease is crucial for understanding the survival and population dynamics of wildlife, since parasites generally have negative effects on host fitness (Ducatez et al. 2020). However, most studies to date have focused on the dynamics of single infectious agents within hosts; this is despite the fact that pathogens and parasites rarely occur in isolation (Lello et al. 2018; Sweeny et al. 2021) and instances of coinfection (also 'mixed infection', 'concomitant infection', 'polyparasitism' (Hoarau et al. 2020), or even 'synzootics' (Sweeny et al., 2021)) raise questions about the mechanisms, evolution, and fitness outcomes (e.g. decreased health, survival) of both transmission of and susceptibility to co-infections. When hosts are more infected by one parasite/pathogen, for example, are they more or less susceptible to other type(s) of infection(s)? And what ecological (e.g., transmission modes, habitat barriers) or host life-history traits (e.g., behavioral, immunological) predict instances of single v. coinfection? Some of these questions have been answered for malarial parasites (Boundenga et al., 2016; Atkinson and LaPointe 2009; Loiseau et al. 2013; Moens et al. 2016; Jia et al. 2018; Su et al. 2020), coccidia (Baeta et al. 2008; Sepp et al. 2011; Václav and Blažeková 2014; Cama and Mathison 2015; Weitzman et al. 2020; Lu et al. 2021), and poxvirus (Piskurek and Okada 2007; McGraw et al. 2020; Williams et al. 2021; Yang et al. 2021) in several different animal groups, but we still largely lack studies in which physiological, health-related metrics and infection status are examined in a multiple parasite system.

Due to the negative effect of parasitism in wildlife, understanding how parasites and pathogens affect physiological processes and health status is of great importance. In springbok antelopes (*Antidorcas marsupialis;* Turner et al., 2012), for example, body condition (BC), which can be considered as a health index for individuals (Peig and Green 2009), was lower in animals with more gastrointestinal parasites (*Strongyloides* sp and *Eimeria* sp.). However, in house sparrows (*Passer domesticus*), infection with haemosporidian parasites positively predicted BC (Jiménez-Peñuela et al. 2019). One possible explanation for the discrepancy in results of these studies is that there are other parasite/pathogen pressures in the animals that were not studied. Several physiological metrics, such as the concentration of antioxidants or leukocyte counts in the bloodstream, have also been used to study links between coinfections and host health state. In Atlantic canaries (*Serinus canaria*), individuals with high levels of vitamin E were less affected by haemosporidian burden (Delhaye et al. 2018), which was a similar pattern found in Ugandan children (Metzger et al. 2001). In addition, the heterophil / lymphocyte ratio, a proxy for stress, was highly associated with co-infections with malarial parasites and microfilariae in white-eyes from New Calendonia (*Zosterops* sp.), whereas in wild dugongs (*Dugong dugong*), H/L and white-blood cell count was not associated with occurrence of two types of blood parasite (*Babesia* and *Anaplasma*; Satyaningtijas et al. 2020). Consequently, the overmentioned studies indicate the parasites affect physiology and health of species throughout many host organisms.

Here, we screened a wild-bird species (the house finch, *Haemorhous mexicanus*) for several avian parasites and pathogens – (a) protozoan parasites: coccidiosis (Eimeriidae: *Isospora* sp.; Berto et al. 2011), haemosporidioses (Haemosporidae: *Plasmodium* sp.; *Haemoproteus* sp.; Valkiunas, 2005), and trichomoniasis (Trichomonadidae: *Trichomonas gallinae* (Alrefaei et al. 2019); (b) viruses: canary poxvirus (Poxviridae: *Avipoxvirus*; Beukema et al. 2006) and west Nile virus (Flaviviridae: *Flavivirus* sp.; Tolsá et al. 2018); and (c) bacteria: mycoplasmal conjunctivitis (Mycoplasmataceae: *Mycoplasma gallisepticum*; Hawley et al. 2018) . House finches has emerged as a model for studies of wildlife disease dynamics (Hill et al., 2004) since it can be infected with several diseases, including mycoplasmosis, coccidiosis, haemosporidioses, poxvirus infections, and west Nile virus (Giraudeau et al., 2014; Hill et al., 2004; Medeiros et al., 2014; Schaper et al., 2021). Prior work in this species showed that severely coccidia-infected house finches developed a higher burden of mycoplasmal conjunctivitis (Weitzman et al. 2020) and that malaria-infected individuals were less likely infected with west Nile virus (Medeiros et al. 2014). However, since we only found infected individuals with coccidiosis and haemosporidioses, we only analyzed both these diseases in the study. House finches are also sexually dichromatic, meaning that males display more elaborate and condition-dependent red, orange, and yellow carotenoid-based plumage colors than females. Carotenoids have multiple physiological functions as well, being an important immune stimulator (Simons et al. 2012), which may be used to fight-off diseases (McGraw et al. 2013), and males infected with mycoplasmal conjunctivitis, coccidia (Brawner III et al.

2000), and poxvirus (Thompson et al. 1997) had drabber plumage compared to uninfected individuals. However, no studies have examined coinfections in relation to ornamental plumage in house finches.

Therefore, our objective was to quantify infection prevalence and severity for two different parasites in individuals from a free-ranging population of house finches and relate these to several metrics of bird body quality and health. More specifically, we aimed to examine interactions between coccidiosis and haemosporidioses, to determine whether the infection by one parasite increases the likelihood of infection of the other. We also assessed the relationship between the physiological metrics and the infection occurrence of haemosporidian parasites and coccidiosis. Based on prior work, we generally predicted that birds with lower circulating carotenoids, vitamins, and body condition, but higher glucose concentrations, global leukocytes, and heterophil/lymphocyte ratios would have a higher burden of parasites/pathogens. Lastly, we also studied male plumage coloration as a predictor of parasite occurrence, hypothesizing that highly infected males would have less red plumage and be in better health and condition compared to non-infected individuals.

5.3 METHODS

5.3.1 Bird measurements and sample collection

From 14-20 October 2021, we captured 55 house finches (22 males, 33 females) in sunflower-seed-baited feeder traps on the Arizona State University campus in Tempe, Arizona, USA. Due to the time of year of this study, when it is difficult to distinguish between hatch-year (HY) and after-hatch-year (AHY) birds based on molt and plumage, fifty of these birds were of unknown age, but we did catch two confirmed hatch-year (HY) males, two hatch-year females, and one after-hatch-year (AHY) female (Pyle 1997); we determined the age of these five birds since they were recaptures from our prior studies. We banded all individuals with a unique numbered US Geological Survey tag for individual identification. We sexed individuals by plumage pattern, with males having red, orange, or yellow carotenoid-containing patches on the crown, rump, and breast (Hill 2002). We measured body mass of each bird to the nearest 0.01 g using a digital scale (Smart Weigh, Chestnut Ridge, NY) and tarsus length to the nearest 0.1 mm using digital calipers. We initially visually inspected all individuals for three different diseases, namely canary poxvirus (we inspected for skin lesions close to the eye, beak and feet; ranking the disease severity with a 0-4 scale, following Giraudeau et al., 2014); mycoplasmal conjunctivitis (*Mycoplasma gallisepticum*; we examined birds for red, swollen eyes; Ley et al., 2018) and trichomoniasis (*Trichomonas* spp.; we opened the beak and searched for cream-colored oral/throat lesions; Tully et al., 2009). We did not find any signs of mycoplasmal conjunctivitis and canary poxvirus in this group of birds, and we observed trichomoniasis lesions in only two individuals, so we did not further consider these three diseases in our analyses and focused only on coccidiosis and malaria (see more below, i.e. we removed the two *Trichomonas*-positive individuals from the dataset).

At capture, we also used a 26-gauge needle to obtain a blood sample through brachial venipuncture; the first two drops were used to measure circulating glucose concentration (mg/dL) using an Accu-Chek Guide glucose monitoring device (Roche Diabetes Care Inc., Indianapolis, IN) and then we collected 80-100 ul whole blood into heparinized capillary tubes, two drops of which were used to make two blood smears (following the protocol described by Valkiūnas 2005) and the rest stored in Eppendorf microtubes, which were centrifuged (10000 rpm for 3 minutes) to separate the plasma from blood cells, both of which were frozen at -80° C until further carotenoid (plasma) and West Nile virus (cells) analyses. We obtained duplicate measures of glucose levels for all birds, and the values were highly repeatable for these individuals (92% repeatability, estimate = 0.97; Std. error = 0.03, $p < 0.01$); so we used average glucose values for each bird in statistical analyses. Here and below, we ran linear models with the *lme4* (Bates et al. 2015) package in R software (R Core Team 2019) for all repeatability analyses.

Males were then transported to a dark room, in which we took digital photos from all males (see below for more information), and then all individuals were kept in small bird cages (26.7 x 21.6 x 34.3 cm) for eight hours in an indoor vivarium with *ad libitum* availability of sunflower seeds and water. At 1700 hrs, since there is a diel shedding cycle of coccidian gametes (Brawner et al. 2000), we collected fresh feces from new cage paper and preserved them in 1 mL of 2.2% potassium dichromate solution. Coccidiosis severity was analyzed using a standard fecal flotation protocol (Brawner et al. 2000) followed by analysis using compound light microscopy (40x magnification; McGraw & Hill 2000). Coccidia severity was estimated using the following 0-5 integer rank scoring system: 0 corresponds to 0 oocysts; 1 = 1-10 oocysts; 2 = 11-100 oocysts; 3 = 101-1000 oocysts; 4 = 1001-10000 oocysts; and 5 > 10000 oocysts. All birds were released back into the wild before sunset at their capture site.

5.3.2 Plumage coloration scoring

We scored male carotenoid-based plumage coloration from six different digital photos (Canon PowerShot SX620 HS, Canon USA, Huntington, NY) taken of each bird - two from each of the three different colorful patches on a male (crown, breast, and rump). Birds were placed against a standard gray board, on which a color-reference photo card was placed (Kodak color strip, Kodak Color Control Patches 2007, NY), and we used consistent flash settings and distance between camera and birds for all photos (Hasegawa et al. 2014). We analyzed photos using Adobe Photoshop CS6 (Adobe System, San Jose, CA, USA), by selecting the color patch with the lasso marquee and using the red-green-blue (RGB) values obtained from the Histogram window (Giraudeau et al. 2013a) to determine hue values using the Color Picker function. Male house finch plumage varies mostly in hue among individuals (McGraw and Hill 2004), and females make mating decisions based on this colorimetric (i.e. prefer redder males; Toomey and McGraw, 2012), so we used hue as our plumage color variable of interest (note: lower hue scores represent redder birds). Two observers measured each photo, and we found very high inter-observer, intra-photo repeatability (96% repeatability, estimate = 0.97; Std. error = 0.03, $p < 0.01$) for hue, so we again used averaged values in statistical analyses.

5.3.3 Blood smears and haemosporidian parasite analyses

On the day of capture, blood smears were fixed using absolute methanol for two minutes, air-dried, and then stained using Giemsa dye for one hour (following Valkiūnas 2005). Duplicate stained slides for each individual were examined under a standard compound microscope at 100x magnification in immersion oil, analyzing 100 fields per slide. One of us (VASP) estimated the number of erythrocytes in all fields and recorded the number of malaria-parasitized red-blood cells (Order Haemosporidae; genera *Plasmodium* and *Haemoproteus*) to calculate parasitemia (proportion of infected erythrocytes out of 10,000 erythrocytes total; Valkiūnas, 2005). The examiner was blind to all physiological and disease occurrence/severity data, knowing only the bird ID at the time of smear analysis. We found only two individuals carrying *Haemoproteus* (two infected erythrocytes in one, and a single infected cell in the second). Both of those birds were also highly infected by *Plasmodium*, so we considered only *Plasmodium* infection as the metric of haemosporidian parasitism in our study. We also estimated the number of white blood cells (lymphocytes, heterophils, eosinophils, basophils, and monocytes) to produce two different variables: heterophil/lymphocyte ratio (H/L) and global leukocytes (hereafter, GL). H/L ratio is a proxy for stress (Gross and Siegel 1983), whereas GL reflects disease progress in birds (Owen and Moore 2006). VAP screened all slides twice. Therefore, we estimated intra-individual repeatability (i.e. between slides from the same individual) using linear models (*lme4* package (Bates et al. 2015) in R software) with the measurements from both slides. All linear models were positive and statistically significant, with moderate to high repeatability for all cell counts (estimate, p-value, and r-squared in parenthesis): GL (66%), heterophil (0.88, < 0.01, 87%), lymphocyte (1.03, < 0.01, 88%), and *Plasmodium* parasites (0.98, < 0.01, 99%).

5.3.4 West-Nile virus analysis

We scored cells to screen for the presence of west-Nile virus (WNV) infection. We did not find any individuals being infected with WNV, so we did not consider this pathogen in our statistical analysis. We followed the same protocol described in Hepp et al. (2018).

5.3.5 Circulating levels of carotenoids and lipid-soluble vitamins

We measured plasma carotenoid and a lipid-soluble vitamin (vitamin E - tocopherol) concentration using high-performance liquid chromatography (HPLC), following McGraw & Toomey (2010). We extracted carotenoids and vitamins with organic solvents (McGraw et al. 2008), centrifuged the solutions for 3 minutes at 10,000 rpm, and transferred the supernatants to a newly labeled tube for nitrogen-streamed evaporation. We then added 200 µL of HPLC mobile phase (42:42:16, methanol:acetonitrile:dichloromethane, v:v:v), vortexed for 2 seconds, and snap-centrifuged to remove any remaining protein. We used Agilent HPLC (Alliance 2695, Waters Corp. Milford, MA, USA) for analysis, and the Open Lab Software version A.01.04 (Agilent Technologies, Santa Clara, CA, USA) to obtain the area under the curve for the following detected compounds: alpha-tocopherol, lutein, and 3'-hydroxy-echinenone. We measured the area under the curve twice for every component and used averaged values for analysis. We estimated the repeatability by adjusting a linear models between both measurements to check for measurement consistency, using the *lme4* package (Bates et al. 2015) in R software. The intra-sample repeatability (between AUC measurements) was as follows (estimate, p-value and r-squared in parenthesis): alpha-tocopherol (0.98, < 0.01, 99%), retinol (0.98, < 0.01, 98%), lutein (1.00, < 0.01, 99%), and 3'-hydroxy-echinenone (0.99, < 0.01, 98%). All linear models were positive and statistically significant. The measurer was blind to all data besides the sample ID at the time of data collection. We calculated concentration (in μg/mL) of each component following McGraw et al. (2013).

5.3.6 Statistical Analysis

We measured body condition for each individual as the residual values from a significant mass-tarsus regression (following McGraw et al. 2020; $F_{1,53} = 13.58$; $p < 0.01$; $R^2 = 0.19$). We also tested the difference between the parasitism by haemosporidian parasites and coccidia between sexes using a linear model, thorugh the lme4 package. To study the relationship between coccidiosis and haemosporidiosis, we tested the correlation between coccidiosis severity and haemosporidian parasite parasitemia with only individuals positive for both infections, using a linear model with the *lme4* package (Bates et al. 2015). We also tested for the association between coccidiosis and haemosporidian parasite occurrence (presence v. absence) using the Cramer's V with the *cramerV* function from the *rcompanion* package (Salvatore Mangiafico 2021). To test for relationships between parasite occurrence and physiological metrics, we used blood-parasite and coccidiosis occurrence as the response variables in separate models, and the following predictors: blood glucose concentration, body condition, GL, HL, and plasma circulating carotenoid levels (alpha-tocopherol [vitamin E], lutein, and 3'-hydroxy-echinenone,). We also included blood-parasite occurrence as a covariate in the coccidia model, and coccidia occurrence in the blood-parasite model. We tested for multicollinearity among predictors by checking the variance inflation factor using the *VIF* function from the *regclass* package (Petrie 2020), and considering a value of two for $\mathrm{GVIF}^{(1/(2*df)}$ as our threshold for multicollinear predictors. We subsequently removed the variables with highest VIF value until the model had variables with $\mathrm{GVIF}^{(1/(2*df)}$ lower than two. In the coccidiosis and haemosporidioses occurrence models, we removed sex ($\mathrm{GVIF}^{(1/(2*df)}= 2.20$ and $\mathrm{GVIF}^{(1/(2*df)} = 2.99$, respectively). For tests examining the effects of physiological predictors (same as above for occurrence models) on severity of haemosporidioses and coccidiosis, we included only positive individuals and ran a generalized linear model using the *lme4* package (Bates et al. 2015). In the coccidiosis severity model, we excluded sex ($\mathrm{GVIF}^{(1/(2*df)} = 2.17$), whereas for haemosporidioses parasitemia we removed the lutein ($\mathrm{GVIF}^{(1/(2*df)} = 3.03$) and 3'-hydroxychinenone ($\mathrm{GVIF}^{(1/(2*df)} = 2.14$). In the haemosporidian parasite parasitemia, we first tested the interaction between sex and all predictors and included results only when such interaction was statistically significant. Finally, to address links between male coloration, disease, and health, we trimmed our dataset to include only males and ran a single model having plumage hue as the response variable, and the following predictors: blood glucose concentration, body condition, GL, HL, plasma circulating carotenoid levels (alpha-tocopherol [vitamin E], lutein, and 3'-hydroxy-echinenone), and blood-parasite and coccidia occurrences. We also tested for multicollinearity in this model and removed haemosporidioses occurrence ($\mathrm{GVIF}^{(1/(2*df)}$ = 2.50). We corrected the explanatory and response variables using logarithmic or square-root functions in R to normalize distributions if necessary. We excluded two individuals with the highest values of parasitemia from all models that included haemosporidian parasite parasitemia, since they were significant outliers and biased the results. We then used an information-theoretic approach (Burnham and Anderson 2002) to test the importance of the explanatory variables in the models. We used the *dredge* function from the *MuMIn* package (Barton 2019) to generate all possible models with the explanatory variables, and used model averaging with *model.avg* function from the *MuMIn* package to calculate the model average estimates (Burnham et al. 2011) for all models whenever the best model did not have a weight higher than 0.8. The best-fitted model was considered as the one with the lowest Akaike Information Criterion (AIC) and with the highest model weight. We selected the most important explanatory variables by assessing the estimate, conditional standard errors, and 95% confidence interval (CI). We used the predicted values for male models to plot statistically significant variables, using the *predict* function from the *stats* package (R Core Team 2019). All analyses were performed in R software (R Core Team 2019).

5.4 RESULTS

5.4.1 Testing co-occurrence of both parasites

We found that 25 finches (45%) were infected with haemosporidian parasites (Figure 1); there was no significant difference in infection rate between females (51% positivity) and males (36% positivity; estimate: -0.81, sd = 0.58, p-value = 0.16). Mean severity (parasitemia) of infected individuals was 8.07 ± 25.99 infected red-blood cells. We found that 34 individuals (61%) had coccidiosis, with no significant difference (Estimate = 0.18, s.e. = 0.58, p-value = 0.75) between occurrence in males (63%) v. females (60%). Of those parasitized, mean estimated coccidian oocyst load was 2.61 ± 0.98. We found 15 individuals being infected by both parasites, nine with only malaria, 19 with only coccidia, and 10 with neither of the parasites. We did not find significant associations between either the occurrence (Cramer's V of 0.03; Figure 2) or severity

(Estimate = 0.13, se = 0.20, p-value = 0.52; Figure 2) of these two parasites in individual birds.

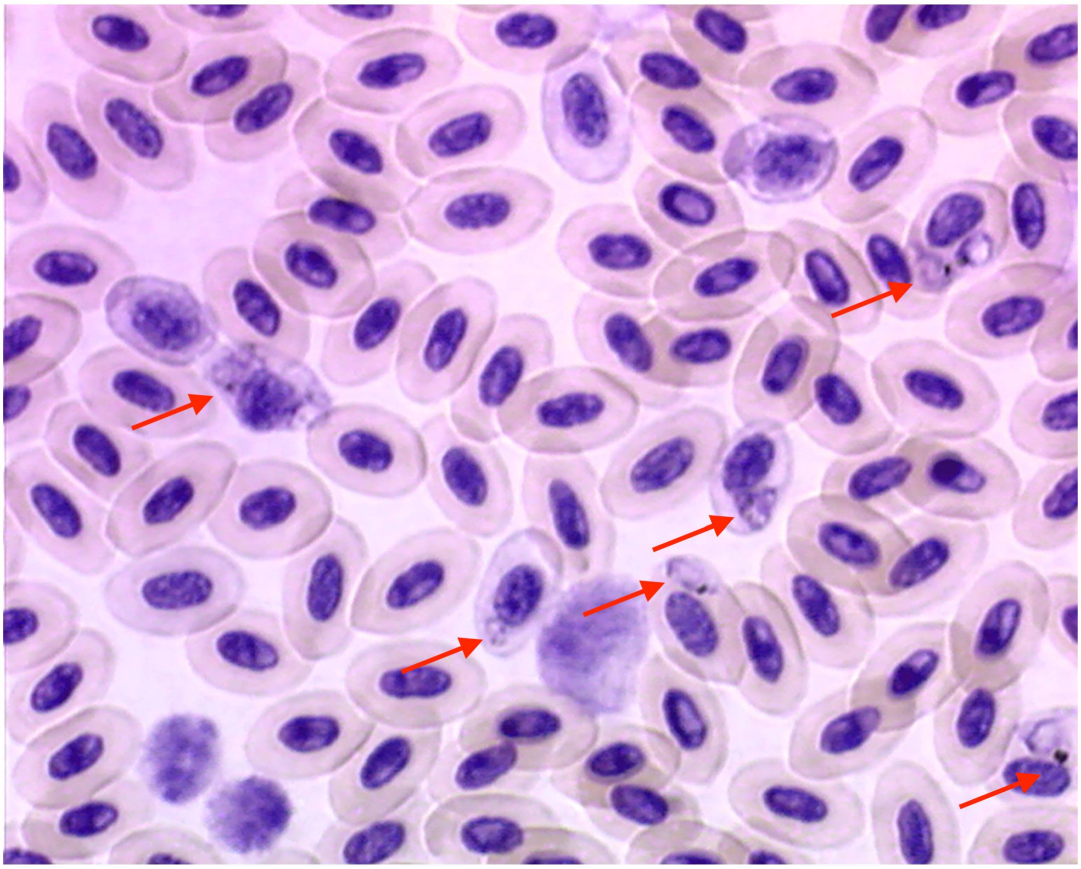

Figure 1: Blood smear from a house finch (*Haemorhous mexicanus*) with infected red-blood cells with *Plasmodium* sp. (Order Haemosporidae), shown with a red arrow.

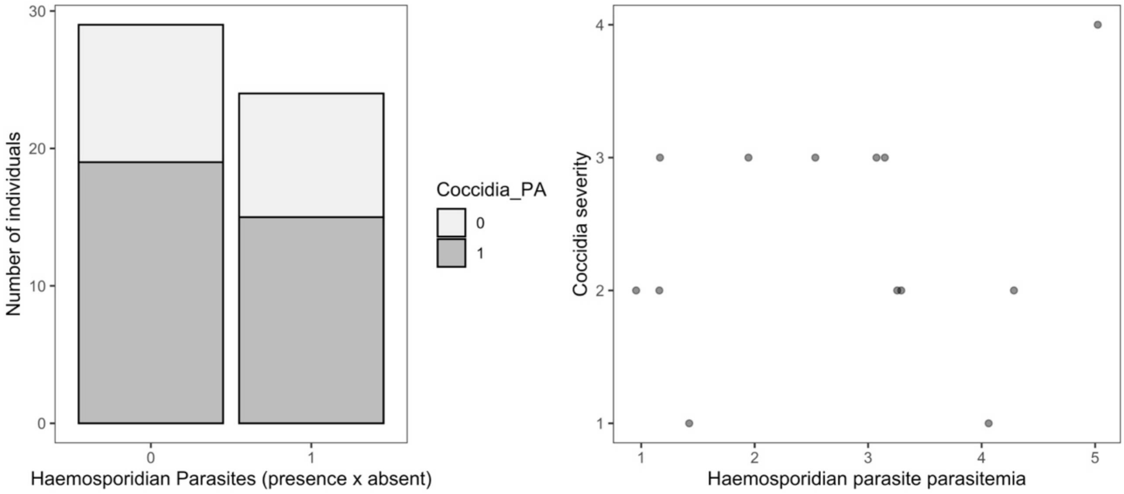

Figure 2: Lack of association between haemosporidian parasite occurrence and coccidia occurrence (Cocidia_PA), with absence (0 values) and presence (1 value) only (left); and coccidia severity and haemosporidian parasite parasitemia (right) – haemosporidian parasite parasitemia can be found in supplemental figure 1..

5.4.2 Relationships between physiological metrics and the occurrence and severity of haemosporidian parasitism

Our model-fitting and -selection procedures revealed a higher H/L and lutein in birds that were infected with haemosporidian parasites (Tables 1, 2, Figure 3). Also, individuals with greater parasitemia of haemosporidian parasitemia had higher blood H/L ratios as well (Table 1, 2; Figure 4).

Table 1: Model selection results for tests of the effects of (A) glucose concentration, body condition, GL, H/L, tocopherol, lutein, 3'-hydroxy-echinenone, and coccidiosis occurrence on haemosporidian parasite occurrence; and (B) glucose concentration, body condition, GL, H/L, tocopherol, and coccidiosis severity on malaria parasitemia. Below we present the variables included in each model, degrees of freedom, AICc values, delta AIC, and weight (*wi*). Here we show the models with delta AIC values lower than two. Sample size for each model is 53 individuals for the occurrence model and 22 for the parasitemia model.

Models	df	AICc	**ΔAICc**	***wi***
(A) Occurrence				
GL + Lutein + H/L	4	64.7	0.00	0.049
Glucose concentration + Lutein + H/L	4	65.8	1.09	0.028
Lutein + H/L	3	65.8	1.13	0.028
Glucose concentration + GL + Lutein + H/L	5	65.8	1.16	0.027
Body condition + Glucose concentration + Lutein + H/L	5	65.9	1.20	0.027
Body condition + GL + Lutein + H/L	5	66.3	1.58	0.022
Body condition + Lutein + H/L	4	66.5	1.80	0.020
GL + Lutein + Tocopherol + H/L	5	66.5	1.85	0.019
(B) Parasitemia				
H/L	3	76.8	0.00	0.210
Sex + H/L	4	78.0	1.18	0.116

Table 2: Model-averaged estimates, standard errors, and 95% confidence intervals of variables in the model using the haemosporidian parasite occurrence (A) and parasitemia (B) as the response variable. Significant variables are marked with an asterisk. Results for 53 individuals for the occurrence model and 22 for parasitemia.

Variables	Estimate	Standard Error	95% C.I.
(A) Occurrence			
Intercept	-7.17	5.52	-18.14, 3.79
GL	1.38	0.76	-0.14, 2.91
Lutein	0.50	0.24	0.01, 0.99*
H/L	1.95	0.94	0.04, 3.85*
Glucose concentration	-0.10	0.00	-0.02, 0.00
Body condition	0.42	0.43	-0.44, 1.29
Tocopherol	-0.22	0.32	-0.88, 0.43
Coccidia occurrence (presence)	-0.27	0.70	-1.68, 1.14
3'-hydroxy-echinenone	-0.37	0.75	-1.89, 1.14
(B) Parasitemia			
Intercept	0.53	1.81	-3.23, 4.31
H/L	2.32	0.66	0.92, 3.72*
Sex (male)	0.80	0.63	-0.52, 2.13
Glucose concentration	0.00	0.00	-0.00, 0.01
Coccidia severity	0.12	0.20	-0.30, 0.55
Body condition	-0.23	0.37	-1.02, 0.55
Tocopherol	-0.18	0.31	-0.84, 0.48
GL	-0.11	0.54	-1.25, 1.02

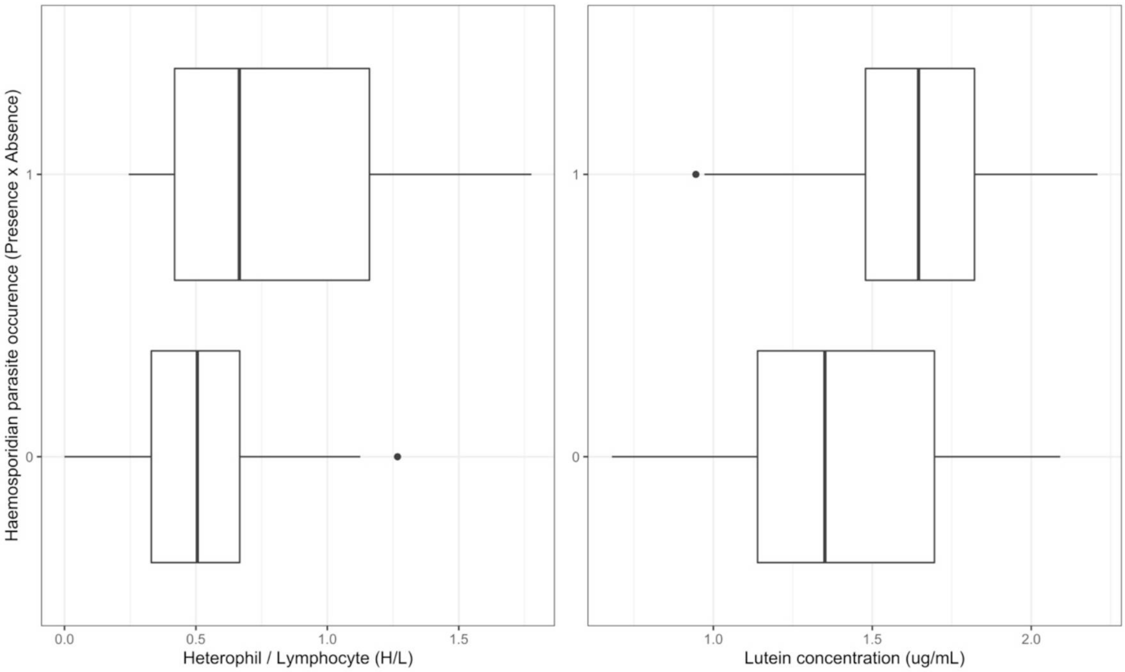

Figure 3: Association between haemosporidian parasite occurrence (presence = 1 ; absence = 0) and heterophil – lymphocyte ratio (H/L) [left], and lutein concentration (right).

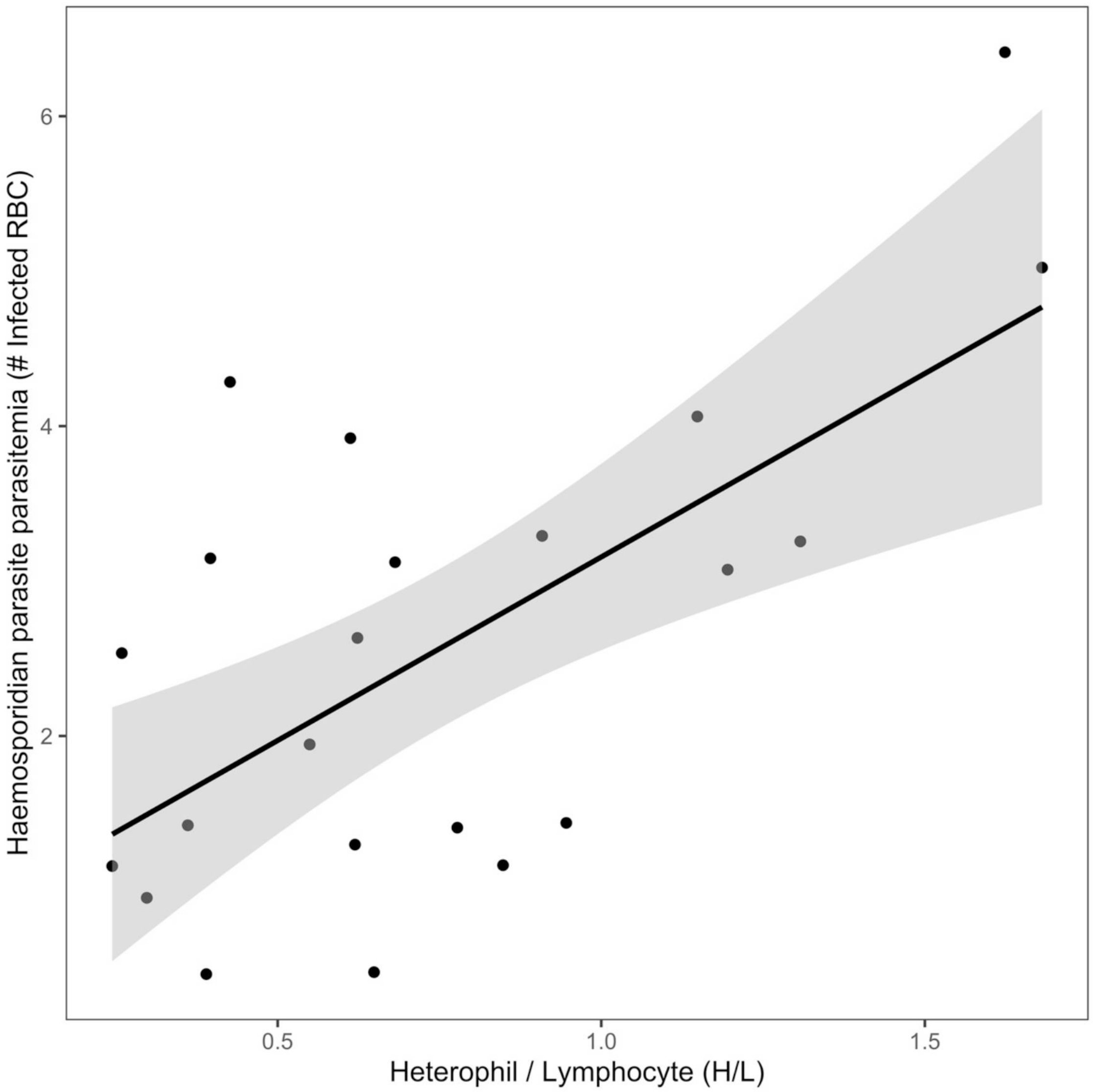

Figure 4: The relationship between haemosporidian parasite parasitemia (number of infected red-blood cells with *Plasmodium* sp) and heterophil – lymphocyte ratio (H/L).

5.4.3 Relationships between physiological metrics and the occurrence and severity of coccidiosis

We did not find support for any of the physiological or health metrics predicting the coccidiosis infection occurrence (Tables 3, 4). However, we found that lower levels of plasma tocopherol (Table 3, 4; Figure 5), predicted severity of coccidiosis. We ran the tocopherol models with and without the potential outlier (indicated with a red arrow) and significance of the results kept the same.

Table 3: Model selection results for tests of the effects of glucose concentration, body condition, GL, H/L, tocopherol, lutein, 3'-hydroxy-echinenone, and haemosporidioses, on coccidiosis occurrence (A) and severity (B). Below we present the variables included in each model, degrees of freedom, AICc values, delta AICc, and weight (*wi*). Here we show the models with delta AIC values lower than two. Sample size for each model is 53 individuals for the occurrence model and 32 for the severity model.

Models	df	AICc	**ΔAIC**	w_i
(A) Occurrence				
Null	1	71.2	0.00	0.047
Body condition	2	72.0	0.74	0.033
Tocopherol	2	72.2	0.98	0.029
Body condition + Tocopherol	3	72.4	1.12	0.027
GL	2	72.6	1.34	0.024
3'-hydroxy-echinenone	2	72.7	1.41	0.023
Glucose concentration	2	73.1	1.86	0.019
Body condition + GL	3	73.1	1.90	0.018
(B) Severity				
Tocopherol	3	86.7	0.00	0.164
Haemosporidioses parasitemia + Tocopherol	4	88.7	1.99	0.061

Table 4: Model-averaged estimates, standard errors, and 95% confidence intervals of variables in the model using the coccidia occurrence (A) and severity (B) as the response variable. Significant variables are marked with an asterisk. Results for 53 individuals for the occurrence model and 32 for parasitemia.

Variables	Estimate	Standard Error	95% C.I.
(A) Occurrence			
Intercept	-1.04	3.36	-7.74, 5.56
Body condition	-0.47	0.36	-1.20, 0.25
Tocopherol	0.41	0.37	-0.34, 1.16
GL	0.66	0.64	-0.62, 1.95
3'-hydroxy-echinenone	-0.54	0.62	-1.79, 0.70
Glucose concentration	0.00	0.00	-0.00, 0.02
Haemosporidioses occurrence	-0.20	0.64	-1.50, 1.10
H/L	-0.30	0.74	-1.79, 1.19
Lutein	-0.03	0.18	-0.41, 0.33
(B) Severity			
Intercept	2.72	1.13	0.39, 5.04*
Tocopherol	-0.48	0.16	-0.82, -0.15*
Haemosporidioses parasitemia	-0.07	0.10	-0.29, 0.14
Body condition	0.09	0.17	-0.26, 0.45
3'-hydroxy-echinenone	0.18	0.39	-0.61, 0.98
GL	-0.15	0.33	-0.83, 0.52
Glucose concentration	0.00	0.00	-0.00, 0.00
H/L	-0.01	0.42	-0.87, 0.85
Lutein	0.01	0.09	-0.17, 0.20

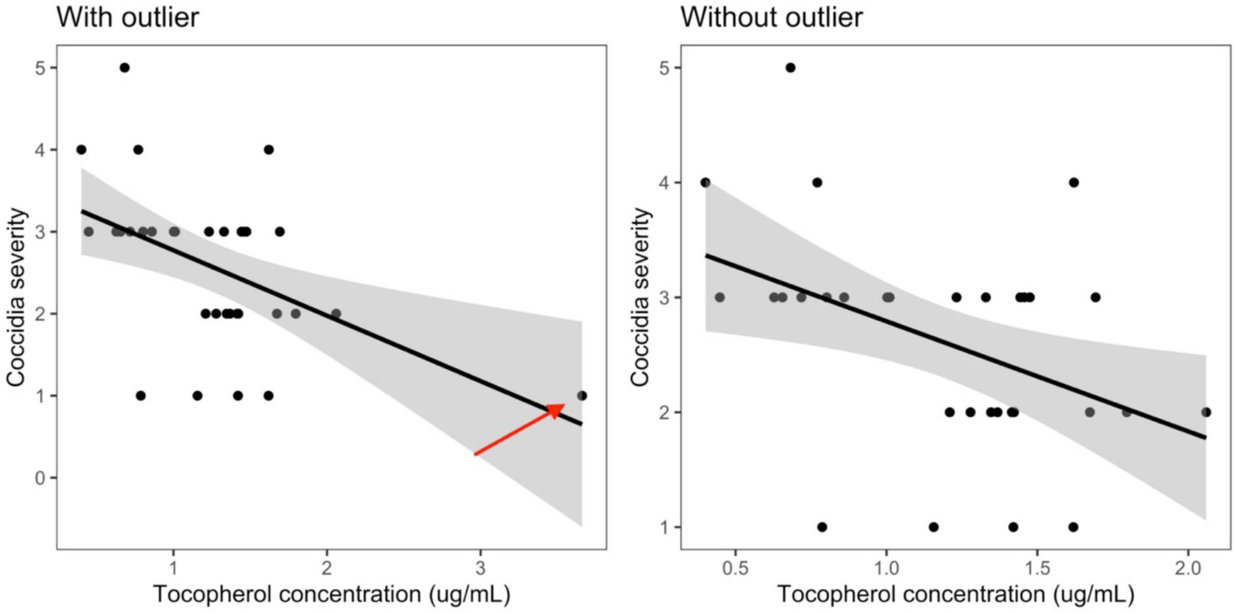

Figure 5: Relationship between coccidiosis severity (estimated on an integer scale from 0-5, with 0 being no infection and 5 being most severe; see text for more details) with (left – indicated with a red arow) and without (right) potential outlier.

5.4.4 Predicting male plumage hue with physiological and disease variables

We found that birds with lower hue scores (redder plumage) had a higher concentration of circulating 3’-hydroxy-echinenone (Tables 5, 6; Figure 6).

Table 5: Model selection results for tests of the effects of glucose concentration, body condition, GL, H/L, tocopherol, lutein, 3'-hydroxy-echinenone, and coccidiosis occurrence on hue of male plumage coloration. Below we present the variables included in each model, degrees of freedom, AICc values, delta AICc, and weight (*wi*). Here we show the models with delta AIC values lower than two. Sample size for each model is 21 individuals.

Models	Df	AICc	**ΔAICc**	w_i
3'-hydroxy-echinenone	3	123.9	0.00	0.205
H/L + 3'-hydroxy-echinenone	4	125.7	1.72	0.087
Body condition + 3'-hydroxy-echinenone	4	125.9	1.93	0.078

Table 6: Model-averaged estimates, standard errors, and 95% confidence intervals of variables in the model using the male hue coloration as the response variable. Significant variables are marked with an asterisk. Results for 21 individual males.

Variables	Estimate	Standard Error	95% C.I.
Intercept	13.70	6.56	0.01, 27.38*
3'-hydroxy-echinenone	-5.62	1.61	-9.01, -2.23*
H/L	2.54	2.18	-2.24, 6.96
Body condition	-1.23	1.22	-3.82, 1.34
GL	1.11	1.95	-2.99, 5.21
Lutein	-0.40	0.83	-2.16, 1.35
Tocopherol	-0.35	1.00	-2.45, 1.78
Glucose concentration	0.00	0.02	-0.05, 0.06
Coccidia occurrence	0.21	2.05	-4.11, 4.54

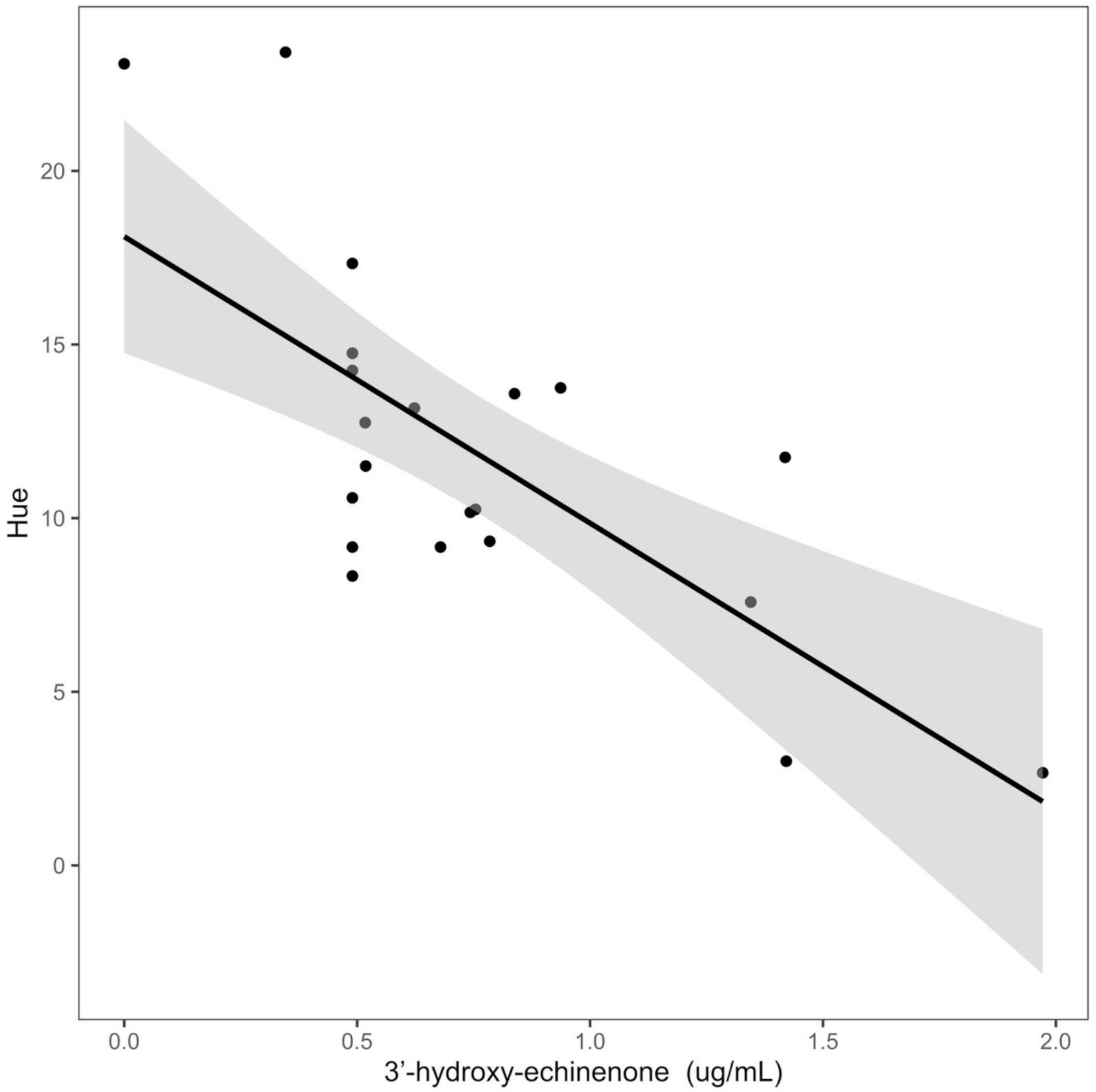

Figure 6: Relationship between hue (lower hue means redder plumage patches on males) and 3’-hydroxy-echinenone (ug/mL).

5.5 DISCUSSION

We found that, despite some level of coinfection, the occurrence and severity of malarial and coccidian parasites were not related in individual birds. We also found that individuals with high levels of H/L and lutein were more likely to have haemosporidian parasite infections. In addition, parasitemia with malarial parasites was positively predicted by H/L ratios, and coccidiosis severity was linked to lower levels of tocopherol. Lastly, we found that redder males had higher levels of the circulating carotenoid 3’-hydroxy-echinenone.

We found that haemosporidian parasitemia and occurrence were not associated with coccidiosis occurrence and severity. This is unlike previous studies in house finches, where haemosporidian parasites have been negatively associated with west-Nile virus infection (Medeiros et al. 2014), but positively related to mycoplasmal conjunctivitis (Dhondt et al. 2017); also house finches severely infected with coccidiosis had more severe cases of mycoplasmal conjunctivitis (Weitzman et al. 2020). *Mycoplasma gallisepticum* appeared in house finch's population in the mid-1990s, a very recent exposure (Luttrell et al. 2001). Therefore, the lack of immunocompetence against a newly acquired parasite may increase susceptibility of already compromised individuals (positive for coccidia). However, western US-house finch populations, the location of our study, has an overall higher haemosporidian parasite prevalence, compared to eastern populations (Davis et al. 2013), which may suggest that malarial parasites have a long immunological history in these populations. On the other hand, coccidia is also a fairly common parasite in birds (Knight et al. 2018), indicating that wild birds may already have established a stable defense against coccidia and malarial parasites, which explains the absence of a significant association between both diseases. Therefore, it looks like that the blood (haemosporidians) and gut (coccidians) parasites analyzed here may have a long immunological history in our house finch populations, with distinct pathways, affecting individuals differently, even though some level of co-infection occurred.

We also found that finches with malaria infection (both occurrence and severity) had higher H/L ratio. High H/L ratios typically are thought to reveal chronic stress, and have been linked with several diseases, such as haemosporidian parasites (Wojczulanis-Jakubas et al. 2012; da Silva Rodrigues et al. 2021), *Hepatozoon* (Wojczulanis-Jakubas et al. 2012), and microfilariae (Clark et al. 2016) in several bird species. Heterophils are highly phagocytic, able to fight-off a broad spectrum of microbes (Harmon 1998), and activated by cytokines and chemokines to strengthen phagocytic ability (Kogut et al. 1993). On the other hand, lymphocytes are mainly related to humoral immunity, increasing antibody production (Sharma 1991). Therefore, we believe that infection by haemosporidian parasites increases immune burden, thus decreasing lymphocytes. Also, the more infected individuals are, the more likely they will be to have an increased H/L ratio, suggesting a higher stress in individuals with higher parasitemia. For example, in both experimentally-infected (Bale et al. 2020) and in naturally acquired infections (Davis et al. 2004; Fratto et al. 2014) in house finches with *Mycoplasma gallisepticum*, a marked decrease of lymphocytes was recorded, suggesting that H/L ratio tend to be higher in infected individuals. We found the same results for *Plasmodium* parasites, meaning that this physiological trait is consistent with different diseases.

We also found that house finches infected with haemosporidian parasites had higher circulating lutein. Lutein has been shown to be associated to the antioxidant activity in birds (Alves-Rodrigues and Shao 2004). For example, supplemented zebra finches (*Taeniopygia guttata*) with lutein and zeaxanthin had higher humoral and cell-mediated immunity compared to individuals without the carotenoid supplementation (McGraw and Ardia 2003). Also, Baeta et al. (2008) studying the relationship between circulating carotenoids in blackbirds (*Turdus merula*) found that coccidia-infected individuals supplemented with carotenoid-rich resources slowed down parasite replication with slower carotenoid assimilation. Therefore, our results suggest that malaria-infected individuals may trade investing lutein in other functions to immunity, and that those individuals harboring haemosporidian parasites may assimilate lutein more slowly compared to uninfected individuals, which may explain the positive association between lutein concentration and haemosporidioses. Alternatively, plasma lutein concentration has also been associated with fat storage in garden warblers (*Sylvia borin*), and once infected with coccidia, the fat reserves immediately dropped down (Metzger and Bairlein 2011). Therefore, infected house finches in our study may be using an internal storage of lutein to fight-off malaria infection, as indicated by the greater availability of plasma lutein compared to uninfected individuals.

Also, house finches with a higher burden of coccidia had lower concentrations of circulating tocopherol. In black siskins (*Spinus atrata*; cardueline-finch relatives of house finches), *Isospora* coccidian gut-parasites can cause vitamin malabsorption due to duodenal and intestinal damage (Giacomo et al. 1997). Our results for plasma vitamin E are consistent with this and, given the strong antioxidant role of tocopherol (Shah et al. 2016), suggest either that (a) finches with severe coccidiosis may also suffer from reduced antioxidant protection, as in broiler chickens (*Gallus gallus domesticus*; Perez-Carbajal et al. 2010; Gautier et al. 2020) and greenfinches (*Carduelis chloris*; Hõrak et al. 2004), or (b) the body pool of tocopherol has been drained to help combat coccidian infection and support associated immune responses. For example, Allen et al. (1997) found that coccidiosis increases free radical production, and depleted vitamin E in the bloodstream. On the other hand, in house finches, tocopherol levels were negatively related to circulating carotenoids (Giraudeau et al. 2013b), suggesting a possible competition between both components, and also that there is a tendency of decreasing its levels after winter (Giraudeau and McGraw 2014). Therefore, we demonstrated that in addition to season and carotenoid, tocopherol also related to the coccidia burden in house finches.

Lastly, we found a negative association between hue and 3’-hydroxy-echinenone, with redder males having more 3’-hydroxy-echinenone. 3’-hydroxy-echinenone is a keto-carotenoid, and it is one of the main precursors of the red coloration in these birds. Birds must metabolically transform yellow carotenoids, such as cryptoxanthin, into red carotenoids through ketolation processes (Hill et al. 2019). Our results are in accordance with other studies, pointing to the key role of 3'-hydroxy-echinenone for plumage redness in house finches (McGraw et al., 2003, 2013).

In summary, our results showed that a blood parasite (*Plasmodium*) was associated to blood parameters and plasma lutein, whereas the gut parasite (coccidia) was only associated to the absorption or differential usage of tocopherol (vitamin E). Finally, redder males had more 3'-hydroxy-echinenone, which is a red-carotenoid that can be an important cue for selecting mates during the reproductive period. Our study demonstrated that infection by coccidia and malaria in urban birds may be common, and that different parasites may affect house finches differently, demonstrating the importance of screening wild birds for multiple parasites in ecological studies.

5.6 LICENSES

This study was performed under the United States Fish and Wildlife Service permit (MB088806-1), United States Geological Survey banding permit (23362) and Arizona State Game and Fish scientific collecting permit (SP406785). This study was also approved by the Institutional Animal Care and Use Committee at Arizona State University (21-1833R).

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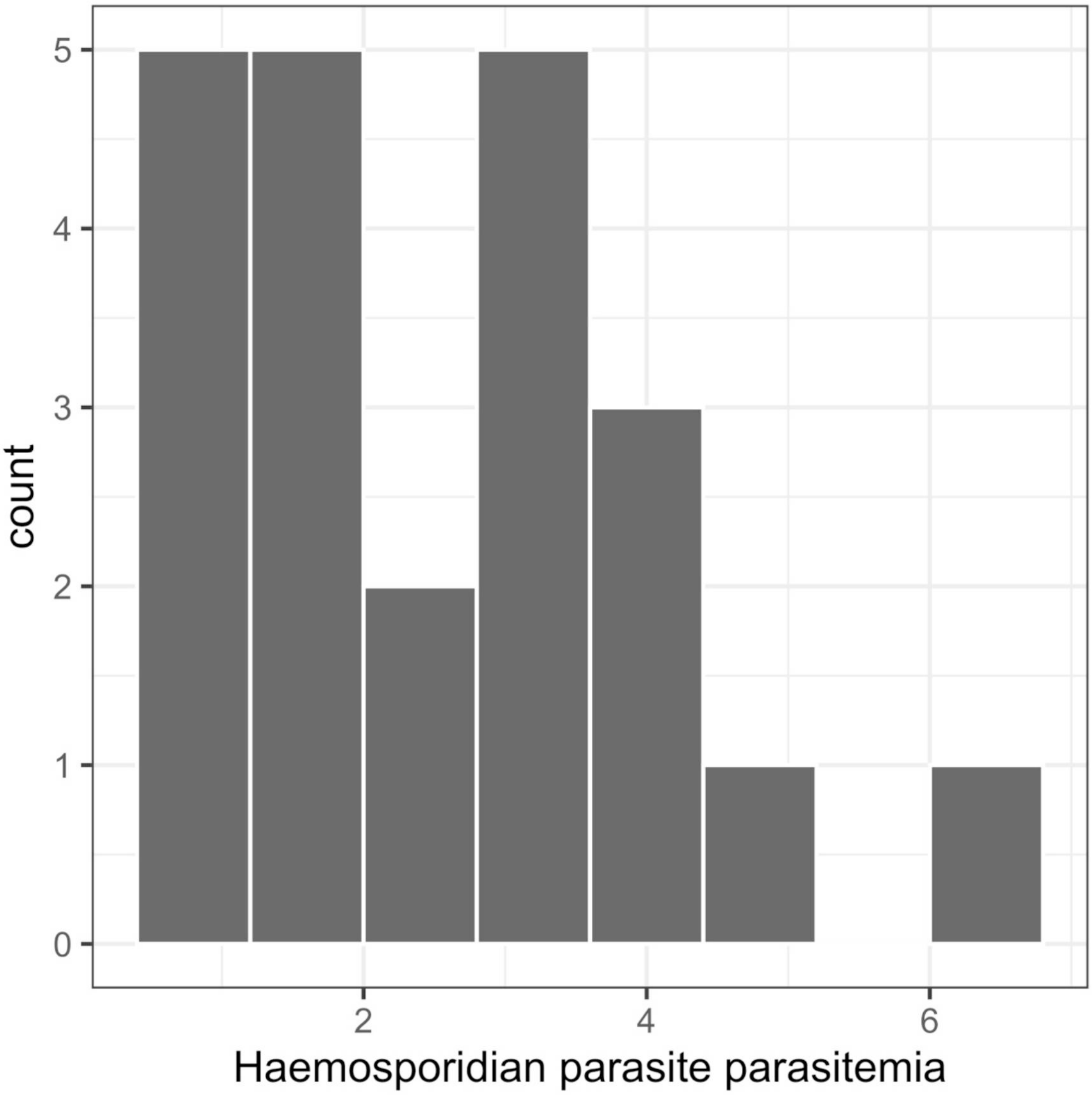
5.8 Supplemental material



Supplemental figure 1: Histogram of the haemosporidian parasite parasitemia in house finches capture at the Arizona State University, Tempe campus in October, 2021.

6.0 Considerações finais

Malária aviária é uma doença transmitida por vetores dípteros (Valkiunas, 2005), podendo ser detrimentais para indivíduos infectados (Ribeiro *et al.*, 2005; Ricklefs and Sheldon, 2007; De La Torre *et al.*, 2020; da Silva Rodrigues *et al.*, 2021), causando até a morte (Jia *et al.*, 2018). Dessa maneira, entender os padrões de distribuição e ocorrência dessa doença é uma importante tarefa para monitorar espécies mais vulneráveis e localidades de maior incidência da doença. Para melhor entender a prevalência de malária aviária, no primeiro capítulo, estudamos essa interação antagonista em traupídeos, uma das maiores famílias de aves neotropicais. Para isso, capturamos 2315 indivíduos de 47 espécies diferentes e testamos todos para a presença de linhagens de *Plasmodium* e *Parahaemoproteus*, ambos gêneros de parasitos causadores de malária aviária. Utilizamos modelos filogenéticos, que levam em consideração o parentesco entre as espécies de hospedeiro, para entender como o clima e traços da história de vida de traupídeos aumentam as chances de infecção por malária aviária. Mais especificamente, testamos a influência da temperatura e precipitação média das localidades onde cada espécie ocorre, além de métricas de história de vida como participação em bandos mistos, tamanho corporal, duração do período de incubação, quantidade de ovos produzidos durante o período reprodutivo, status de migração (migrante x não migrante), altura de ninho e forrageamento, cobertura vegetal e dieta sobre a prevalência de parasitos. Observamos que a temperatura influência de forma diferente cada gênero de malária aviária, sendo o *Plasmodium* mais prevalente em espécies que ocorrem em ambientes com maior média de temperatura anual, e o *Parahaemoproteus* mais prevalente em espécies provenientes de localidades com menor temperatura média anual. Esse é um importante resultado, uma vez que entender os padrões de ocorrência dessa doença em um cenário de aquecimento global, pode auxiliar projetos de conservação a identificar quais espécies podem ser reservatórios de doenças, além de predizer quais espécies podem ser mais vulneráveis. Além disso, espécies que incubam por um período maior também são mais acometidas por *Parahaemoproteus*. Acreditamos que incubar por mais tempo pode aumentar a probabilidade de encontrar vetores infectados por malária, uma vez que espécies que perduram no espaço por mais tempo, podem ser alvo mais frequente de vetores. Encontramos também que espécies provenientes de habitats com menor cobertura vegetal são mais prevalentes para *Parahaemoproteus*. Esse resultado nos dá um indicativo de que habitats mais abertos podem ser fonte de vetores transmissores desse gênero, aumentando a prevalência da doença. Por fim, encontramos que espécies que se juntam a bandos mistos também são mais acometidas por parasitos causadores de malária do gênero *Plasmodium*. Esse resultado sugere que a participação em bandos pode aumentar as chances de identificação dessas aves por vetores da doença, seja por pistas olfatórias ou visuais. Além disso, bandos tendem a cobrir uma parte maior do habitat, aumentando sua chance de encontro com vetores, e por consequência, vetores infectados. Nossos resultados para esse capítulo mostram, portanto, que em um cenário de aquecimento global, é possível que a prevalência da doença pode ser significativa tanto em áreas que irão aquecer quanto em áreas que irão resfriar. Os traços da história de vida de traupídeos também foram importantes para explicar a prevalência de parasitos causadores de malária, demonstrando que estar em um espaço por mais tempo (incubação), agregar-se a outros indivíduos no mesmo espaço (bandos mistos) e habitar locais abertos aumenta a chance de encontro com vetores infectados. De qualquer forma, ao compreender melhor a distribuição e ocorrência da doença, tentamos entender quais são as consequências do parasitismo para as características secundárias para essas espécies.

Entender como parasitos causadores de malária influenciam características secundárias de aves, podem nos dar pistas de como relações antagonistas influenciam a escolha de parceiros durante a reprodução, já que aves são organismos muito visuais (Espmark *et al.*, 2000). Assim, no segundo capítulo, estudamos a relação entre a prevalência de parasitos causadores de malária aviária com a coloração de plumagem. A coloração de plumagem é proveniente basicamente de pigmentos ou de coloração estrutural. Pigmentos tem múltiplos papeis na fisiologia de aves, como estimulantes imunes, reserva energética, além da deposição de pigmentos em penas, o que origina a coloração da plumagem (Hill and McGraw, 2006). Assim, para entender essa relação testamos 4232 indivíduos de 53 espécies da família Thraupidae para a presença de *Plasmodium* e *Parahaemoproteus*. Usamos dados previamente publicados para as seguintes informações: coloração de plumagem, história de vida, e filogenia dos hospedeiros. Utilizamos novamente modelos filogenéticos para levar em consideração a relação de parentesco entre as espécies de aves. Vimos que espécies dicromáticas, ou seja, espécies em que o macho e a fêmea têm traços de coloração distintos, possuem uma maior tendência de serem parasitadas. Essa relação pode demonstrar que o parasitismo influencia na escolha dos parceiros, uma vez que o parceiro que escolhe pode privilegiar indivíduos que tenham plumagens mais conspícuas, e, portanto, melhor condição. Encontramos esse mesmo padrão também para os modelos de complexidade da coloração de machos e fêmeas. Machos e fêmeas mais complexos em termos de coloração de plumagem são mais parasitados e tem maior riqueza de linhagem de parasitos, respectivamente. Por fim, demonstramos que espécies com menor tamanho corporal também são mais dicromáticas. Nosso resultado é um indicativo de que espécies menores sofrem maior pressão de seleção sexual. Levando em consideração os dois primeiros capítulos, nós demonstramos quais são as espécies que tem maior probabilidade de infecção, e, portanto, maior vulnerabilidade. Consequentemente, espécies com maior pressão pelo parasitismo também devem sofrer maior pressão seletiva no momento de escolha de parceiros, mostrando a importância de relações antagonistas para a seleção sexual em traupídeos. No entanto, o parasitismo também pode estar conectado com diversas outras métricas fisiológicas, como hormônios de estresse e índice de condição corporal.

Parasitos causadores de malária aviária, hormônio de estresse como a corticosterona, a condição corporal e a coloração da plumagem são métricas previamente utilizadas por diversos trabalhos para compreender a condição e o status de saúde de organismos (Hõrak *et al.*, 2004; Hasselquist, 2007; McGraw *et al.*, 2013; Wang *et al.*, 2013; Granthon and Williams, 2017; De La Torre *et al.*, 2020). Portanto, entender como essas variáveis se relacionam em populações selvagens de aves nos permite entender os impactos na saúde de aves. Assim, para isso utilizamos capturas de aves feitas na Reserva Mananciais da Serra em Piraquara, Paraná, Brasil. Utilizamos duas espécies foco, o pula-pula assobiador (*Myiothlypis leucoblephara*) e o chupa-dente (*Conopophaga lineata*). Nós testamos todos os indivíduos para a presença de parasitos causadores de malária aviária (Haemosporidae, gêneros *Plasmodium*, *Parahaemoproteus* e *Leucocytozoon*). Fizemos ensaios de ELISA para quantificar a concentração de corticosterona, o principal hormônio de estresse em aves (Bortolotti *et al.*, 2008), em penas de voo de ambas as espécies. Além disso, utilizamos um índice que relaciona a massa corporal e o comprimento da asa como o índice de condição corporal, que é uma aproximação do status nutricional dos indivíduos. Como as relações entre coloração de plumagem, condição corporal, ocorrência de parasitos causadores de malária e concentração de corticosterona em penas já foram demonstradas anteriormente na literatura, utilizamos modelos de equações estruturais, que nos permite testar múltiplas hipóteses ao mesmo tempo. Não encontramos suporte para a condição corporal predizer ou ser prevista por nenhuma outra variável, mas o parasitismo por malária foi positivamente correlacionado com a concentração de corticosterona em penas do pula-pula assobiador. Como a definição do momento exato da infeção nessas populações naturais não pôde ser feita, entendemos que essa relação pode ser explicada por duas hipóteses: 1) indivíduos infectados estão mais estressados, e por conseguinte aumentaram a produção de corticosterona, ou 2) indivíduos já previamente estressados e com alta exposição prolongada a corticosterona são imunossuprimidos e são mais vulneráveis a infecção por malária aviária. De qualquer maneira, essa relação é importante, pois demonstra como o parasitismo está intrinsecamente relacionado ao estresse nessa população selvagem do pula-pula assobiador. Por outro lado, encontramos que indivíduos de chupa-dente parasitados por malária tiveram plumagens do peito com menor saturação. Essa relação pode demonstrar que indivíduos infectados são menos capazes de procurar e assimilar recursos ricos em pigmentos para coloração de plumagem, ou que indivíduos infectados investem mais em pigmentos assimilados para a estimulação da resposta imune e menos em coloração da plumagem. Assim, mostramos que o parasitismo por parasitos causadores de malária aviária tem uma importante relação com estresse, além de estarem mais frequentemente associadas com indivíduos com menor saturação na plumagem. No entanto, o parasitismo por uma única doença é raramente encontrado, sendo que o mais provável é que diversos parasitos ocorram mutuamente em populações livre de aves.

Assim, o último capítulo estudou a relação entre parasitos causadores de malária (*Plasmodium* somente) e parasitos intestinais (*Isospora* sp.), além de entender quais parâmetros fisiológicos melhor explicam a ocorrência de cada doença no organismo de aves. Para isso, utilizamos o pintarroxo-caseiro (*Haemorhous mexicanus*) como espécie modelo, sendo que os indivíduos foram capturados no campus da Arizona State University, Tempe, Arizona, Estados Unidos da América. Mais especificamente, testamos se a ocorrência e severidade das doenças estão mutuamente conectadas, além de testar se parâmetros hematológicos (leucócitos globais e índice heterófilos / linfócitos – H/L), condição corporal, vitaminas (tocoferol – vitamina E) e carotenoides circulantes (luteína e 3-hidroxi-equinenona) variam em função do parasitismo. Não encontramos evidência para a coinfecção das doenças, mesmo que algum grau tenha acontecido. Vimos também que a ocorrência e parasitemia de *Plasmodium* foi positivamente explicada pelo H/L, indicando que indivíduos mais estressados tem maior probabilidade de infecção e maior severidade da doença. Além disso, vimos que indivíduos infectados por malária aviária tiveram maior concentração de luteína na corrente sanguínea. A luteína é um importante estimulante imune, que pode aumentar a resposta humoral em indivíduos (McGraw and Ardia, 2003). Em momento de infecção, aves podem utilizar reservas de luteína para estimular o sistema imune de forma mais lenta (Baeta *et al.*, 2008), o que pode explicar a relação positiva entre concentração plasmática de luteína e a ocorrência de infecção por *Plasmodium*. Vimos também que a concentração de tocoferol explicou negativamente a severidade da doença causada por parasitos intestinais. É sabido que parasitos do gênero *Isospora* podem danificar as paredes do duodeno e intestino, acarretando uma redução de absorção de vitaminas no trato intestinal (Giacomo *et al.*, 1997). Assim, indivíduos da espécie pintarroxo-caseiro que tinham uma severidade de *Isospora* maior no intestino podem absorver menos tocoferol, e/ou devem estar utilizando as reservas para combater o parasitismo por essa doença, o que explica a relação negativa entre severidade de coccídea e tocoferol. Por fim, encontramos que machos que tinham mais 3-hidroxi-equinenona circulante aparentavam ter uma plumagem mais avermelhadas. Esse resultado corrobora a relação importante da 3-hidroxi-equinenona como um dos mais importantes pigmentos para coloração vermelha para essa espécie, e que machos que conseguem adquirir recursos ricos com esse carotenoide, conseguem obter uma plumagem mais avermelhada.

De maneira geral, nossos resultados demonstram que espécies dicromáticas são potenciais reservatórios de doenças, e devem ser levadas em consideração em projetos que tentam identificar espécies vulneráveis em projetos de conservação. Além disso, adicionar a identificação de parasitos em estudos ecológicos podem auxiliar na melhor compreensão dos processos fisiológicos e morfológicos de espécies. Consequentemente, uma vez que entendemos melhor como as interações antagonistas afetam populações livres de aves, podemos tomar melhores decisões sobre como agir para proteger espécies ameaçadas.

6.1 Referências

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7.0 APÊNDICES

7.1 General introduction (translated to English)

Haemosporidian parasites occur in several bird species (Agosta *et al.*, 2010), from different habitats, so that antagonistic associations are common in communities (Cable *et al.*, 2017). As parasites can negatively affect hosts, understanding the distribution and occurrence patterns is an important task to better understand disease cycles (Ducatez *et al.*, 2020). For example, malarial parasites are associated with birds with less conspicuous plumage coloration (Romano *et al.*, 2019; Penha *et al.*, 2020a), reduced body condition (Himmel *et al.*, 2020), and can even cause mortality (Permin and Juhl, 2002; Atkinson and Samuel, 2010; Jia *et al.*, 2018). Malarial parasites (Order Haemosporidae, genera *Plasmodium*, *Haemoproteus*, *Parahaemoproteus* and *Leucocytozoon*) are protists with two different phases in their cycle, a sexual one, which occurs in vectors, and an asexual one, which occurs in vertebrates such as birds and mammals. Each genus has a specific group of dipteran vectors that is responsible for carrying the parasites to the vertebrates, with mosquitoes (Culicidae) as vectors of *Plasmodium*, flies (Hippoboscidae) of *Haemoproteus*, louse flies (Ceratopogonidae) of *Parahaemoproteus*, and black flies (Simuliidae) of *Leucocytozoon* (Santiago-Alarcon *et al.*, 2012). Exposure to vectors can be mainly influenced by two factors, climate, and life history traits of hosts. Higher temperatures and precipitation can create optimal conditions for vector development, thus increasing the prevalence of malarial parasites in birds (Loiseau *et al.*, 2013). On the other hand, larger body sizes, higher nesting sites and foraging in the forest stratum, participation in same- or mixed-species flocks, and migration may increase the chances of individuals becoming infected with avian malaria, as these conditions increase the chances of getting into contact with infected vectors (Garvin and Greiner, 2003; Laporta *et al.*, 2011; Ibañez-Justicia and Cianci, 2015; Lutz *et al.*, 2015; Svensson-Coelho *et al.*, 2016). Although in recent decades there has been a growing understanding of the spatial-temporal distribution of avian malaria, there is still a need to better understand the disease distribution in the Neotropical region, a region of high diversity of birds and parasites.

Once infected, birds may trade investing more in the immune response than in other physiological demands, such as plumage color and energy storage (Hill *et al.*, 1999). Plumage coloration comes primarily from two sources: structural coloration or pigments (Hill and McGraw, 2006). Structural coloration is caused by the differential reflection of light on the feather due to differences in pigment-cell format and distribution (Maia *et al.*, 2009). On the other hand, pigments, mostly carotenoids and melanin, come from diet and amino acid metabolism, respectively (Hill and McGraw, 2006). The carotenoid, for example, in addition to plumage coloration, can also be used as an immune stimulator, increasing humoral and cellular immunity in birds (Hill and McGraw, 2006). Thus, infected individuals with haemosporidian parasites appear less conspicuous, which may interfere with the process of choosing sexual partners during reproductive periods (Hamilton and Zuk, 1982). Most studies have attempted to understand how haemosporidian parasites, either individually or at the community level, affect plumage coloration (Figuerola and Green, 2000; Hõrak *et al.*, 2001; Wakamatsu *et al.*, 2013; Dias *et al.*, 2016; Romano *et al.*, 2019; De La Torre *et al.*, 2020; Penha *et al.*, 2020). Thus, there are still few macroecological studies to understand how the pattern of dichromatism, that is, the difference in coloration between males and females, is influenced by haemosporidian parasites. Thus, if mates choose individuals with more conspicuous plumage, we can expect dichromatic species to be more parasitized. Thus, we still require responses to questions such as: dichromatic birds, which are therefore under greater pressure of sexual selection, are more parasitized than monochromatic birds? In any case, the presence of parasitism in birds can influence, in addition to metabolism and plumage coloration, the stress process in organisms.

Stress is considered as any change in organism homeostasis (Romero, 2012). In birds, stress regulation occurs mainly through the action of corticosterone, a hormone controlled by the hypothalamus – pituitary – adrenal gland axis. Corticosterone production occurs in times of stress, such as food deprivation, presence of predators and parasites (Bortolotti *et al.*, 2008). Consequently, higher corticosterone promotes behavioral changes to increase the probability of survival of stressed individuals. However, a prolonged exposure to the corticosterone can cause detrimental effects to birds (Butler *et al.*, 2010). In addition to corticosterone, there are other parameters that serve as an approximation to stress indices, such as the ratio between heterophils and lymphocytes (H/L). Heterophils are used to fight-off a wide spectrum of microorganisms (Harmon, 1998), in addition to activating cytokines to increase the power of phagocytosis (Kogut *et al.*, 1993). On the other hand, lymphocytes are related to humoral defense, increasing antibody production (Sharma, 1991). Thus, high H/L is often related as a measure of chronic stress, such as an increase in infection with malarial parasites (da Silva Rodrigues *et al.*, 2021). However, studies involving several condition metrics related to stress, such as plumage color, corticosterone production, body condition are still few within wild bird populations.

The objective of this thesis was to study the relationship between haemosporidian parasites and birds, in four chapters. The chapters are divided by the ecological scale, with two being macroecological and two local studies. Macroecological studies sought to elucidate the parasite-host relationship considering both parasite and host characteristics. The first chapter aimed to study the effect of climate and life history traits of tanagers (Passeriformes: Thraupidae) on the prevalence of haemosporidian parasites (*Plasmodium* and *Parahaemoproteus*). The second chapter studied the effects of high prevalence and lineage richness of malarial parasites on sexual dichromatism and plumage coloration complexity of tanager species. For the first and second chapters, we used data from birds captured from several countries in the Neotropical region, from southern Mexico to Argentina. All captured individuals were screened for the presence of parasites, genus *Plasmodium* and *Parahaemoproteus*. In both chapters, phylogenetic generalized least square models (PGLS) were made, which consider the phylogenetic proximity among species. For the third and fourth chapters, studies were carried out in the Mananciais da Serra Reserve, Piraquara, PR, Brazil and in the university campus of Arizona State University, Tempe, AZ, United States of America. In the third chapter, we studied the relationship between the feather corticosterone, the occurrence of haemosporidian parasites, body condition, and plumage coloration in two species: white-browed warbler (*Myiothlypis leucoblephara*, Passeriformes: Parulidae) and the rufous gnateater (*Conopophaga lineata*, Passeriformes: Conopophagidae). In this chapter, a path analysis was performed, which allows testing multiple relationships among variables, since these studied variables can influence one another. Finally, the last study was carried out in the house finch (*Haemorhous mexicanus*, Passeriformes: Fringillidae). Since parasites rarely occur in isolation (Sweeny *et al.*, 2021), we studied the effects of *Plasmodium* infection and coccidiosis in individuals from a free-ranging population by extracting several physiological metrics, such as the H/L ratio, global leukocytes, body condition, carotenoids and circulating vitamins. Our study contributed to a better understanding of the factors that influence the occurrence of avian malaria in tanagers, in addition to studying how the high prevalence of parasites influences the evolution of host secondary traits, suggesting a modulatory effect of parasitism in sexual selection. In addition, we studied the relationship between the occurrence of haemosporidian parasites with corticosterone, and the occurrence of parasites and plumage coloration. Finally, we showed how infection by two different parasites can be influenced by different physiological mechanisms. Thus, this study reinforces the recurrent testing of diseases in wild birds as a possible identifier of vulnerable species.

7.2 References

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7.3 Final considerations (translated to English)

Avian malaria is a disease transmitted by dipterans (Valkiunas, 2005), which can be harmful to individuals (Ricklefs and Sheldon, 2007; De La Torre *et al.*, 2020; Ribeiro *et al.*, 2020; da Silva Rodrigues *et al.*, 2021), and may even cause death (Jia *et al.*, 2018). Thus, understanding the patterns of distribution and occurrence of this disease is an important task to monitor the most vulnerable species and locations with the highest incidence of haemosporidian parasites. To better understand the prevalence of avian malaria, in the first chapter, we studied this antagonistic interaction in tanagers, one of the largest families of Neotropical birds. For this, we captured 2315 individuals belonging to 47 different species and screened them all for the presence of *Plasmodium* and *Parahaemoproteus* genera. We used phylogenetic models, which consider the relatedness among host species, to understand how the climate and life history traits of tanagers is associated with the chances of avian malaria infection. More specifically, we tested the influence of temperature and annual average precipitation, as well as life history traits such as participation in mixed-species flocks, body size, incubation period length, clutch size, migration status (migrant x resident), nesting and foraging height, vegetation cover and diet on the prevalence of haemosporidian parasites. We observed that temperature influences each genus of avian malaria differently, with *Plasmodium* being more prevalent in species that occur in environments with higher annual temperature, and *Parahaemoproteus* more prevalent in species from locations with lower temperatures. This is an important result, since understanding the patterns of occurrence of this disease in a scenario of global warming can help conservation projects to identify which species may be reservoirs of diseases, as well as to predicting which species may become more vulnerable. In addition, species that incubate for a longer period are also more affected by *Parahaemoproteus*. We believe that incubating for a longer time may increase the probability of finding infected vectors, since species that linger in space for a longer time may be more frequently targeted by vectors. We also found that species from habitats with less vegetation cover are more prevalent for *Parahaemoproteus*. This result gives us an indication that more open habitats can be a source of vectors that transmit this genus, increasing the prevalence of the disease. Finally, we found that species that join mixed flocks are also more affected by *Plasmodium*. This result suggests that participation in flocks may increase the chances of identifying these birds by disease vectors, either by olfactory or visual cues. In addition, flocks tend to cover a greater part of the habitat, increasing their chance of encountering infected vectors. Our results for this chapter therefore show that in a global warming scenario, it is possible that disease prevalence could be significant in both areas that will warm and areas that will cool down. Tanager life history traits were also important to explain the prevalence of haemosporidian parasites, demonstrating that being in a space for a longer time (incubation), joining other individuals in the same space (mixed flocks) and inhabiting open places, increases the chance of encountering infected vectors. Anyway, by better understanding the distribution and occurrence of the disease, we tried to understand what the consequences of the parasitism in the secondary traits of tanagers are.

Understanding how haemosporidian parasites influence secondary characteristics of birds can give us clues as to how antagonistic relationships influence mate choice during reproduction, as birds are very visual organisms (Espmark *et al.*, 2000). Thus, in the second chapter, we studied the relationship between the prevalence of haemosporidian parasites and plumage coloration. Plumage coloration is basically derived from pigments or structural coloration. Pigments have multiple roles in the physiology of birds, such as immune stimulator, energy storage, in addition to the deposition of pigments in feathers, which gives rise to plumage coloration *per se* (Hill and McGraw, 2006). Thus, to understand this relationship, we tested 4232 individuals from 53 species of the Thraupidae family for the presence of *Plasmodium* and *Parahaemoproteus*. We used previously published data for the following information: plumage coloration, life history traits, and host phylogeny. We again used phylogenetic models to consider the relatedness among bird species. We have seen that dichromatic species, that is, species in which the male and female have distinct coloration traits, have a greater tendency to be parasitized. This relationship can demonstrate that parasitism influences the choices of selecting mates in the breeding season, since the partner that chooses may favor individuals that have more conspicuous plumage, and therefore, better condition. We also found this same pattern for the color complexity models of males and females. More complex males and females in terms of plumage coloration are more parasitized and have greater parasite lineage richness, respectively. Finally, we found that species with smaller body sizes are also more dichromatic. Our result is an indication that smaller species suffer greater pressure from sexual selection. Considering the first two chapters, we demonstrate which species are most likely to be infected and, therefore, most vulnerable. Consequently, species with greater pressure for parasitism must also suffer greater selective pressure when choosing partners, showing the importance of antagonistic relationships for sexual selection in tanagers. However, parasitism may also be connected to numerous other physiological metrics, such as stress hormones and body condition index.

Haemosporidian parasites, stress hormones such as corticosterone, body condition and plumage coloration are metrics previously used by several studies to understand the condition and health status of organisms (Saks *et al.*, 2003; Hasselquist, 2007; McGraw *et al.*, 2013; Wang *et al.*, 2013; Granthon and Williams, 2017; De La Torre *et al.*, 2020). Therefore, understanding how these variables relate to one another allows us to comprehend the several metrics related to health status in birds. Thus, for this, we used bird captures made in the Mananciais da Serra Reserve in Piraquara, Paraná, Brazil. We used two focus species, the white-browed warbler (*Myiothlypis leucoblephara*) and the rufous gnateater (*Conopophaga lineata*). We tested all individuals for the presence of haemosporidian parasites (Haemosporidae, genera *Plasmodium*, *Parahaemoproteus* and *Leucocytozoon*). We performed ELISA assays to quantify the concentration of feather corticosterone, the main stress hormone in birds (Bortolotti *et al.*, 2008). In addition, we used an index that relates body mass and wing length as the body condition index, which is a proxy of the nutritional status of individuals. As the relationships between plumage color, body condition, occurrence of haemosporidian parasites and corticosterone concentration in feathers have already been demonstrated in the literature, we used structural equation models, which allow us to test multiple hypotheses at the same time. We found no support for body condition to predict or be predicted by any other variable, but malarial parasite occurrence was positively correlated with corticosterone concentration in white-browed warblers. As the definition of the exact moment of infection in these natural populations could not be identified, we understand that this relationship can be explained by two hypotheses: 1) infected individuals are more stressed, and therefore increased corticosterone production, or 2) individuals who had previously been stressed by any other source and with high prolonged exposure to corticosterone are immunosuppressed and are more vulnerable to avian malaria infection. Either way, this relationship is important, as it demonstrates how parasitism is intrinsically related to stress in this wild population white-browed warblers. On the other hand, we found that infected individuals of rufous gnateaters had lower breast plumage saturation. This relationship may demonstrate that infected individuals are less able to seek and assimilate carotenoid-rich resources, or that infected individuals invest more in immune response stimulation and less in plumage coloration. Thus, we showed that parasitism by haemosporidian parasites has an important relationship with stress, in addition to being more frequently associated with individuals with lower plumage saturation. However, parasitism by a single disease is rarely found, and it is more likely that several parasites mutually occur in free populations of birds.

Thus, the last chapter studied the relationship between haemosporidian parasites (*Plasmodium* only) and intestinal parasites (*Isospora* sp.), in addition to understanding which physiological parameters best explain the occurrence of each disease in birds. For this, we used the house finch (*Haemorhous mexicanus*) as a study species, and the individuals were captured on the campus of Arizona State University, Tempe, Arizona, United States of America. More specifically, we tested whether the occurrence and severity of diseases are mutually connected, in addition to testing whether hematological parameters (global leukocytes and heterophil / lymphocyte ratio – H/L), body condition, vitamin (tocopherol – vitamin E) and circulating carotenoids (lutein and 3-hydroxy-echinenone) vary as a function of parasitism. We found no evidence for one disease increasing the likelihood of infection of the other, even though some degree of co-infection has occurred. We also found that the occurrence and parasitemia of *Plasmodium* was positively explained by H/L, indicating that more stressed individuals have a greater probability of infection and greater disease severity. In addition, infected individuals with haemosporidian parasites had higher concentration of lutein in the bloodstream. Lutein is an important immune stimulant, which can increase the humoral response in individuals (McGraw and Ardia, 2003). At the time of infection, birds can use lutein reserves to stimulate the immune system more slowly (Baeta *et al.*, 2008), which may explain the positive relationship between plasma lutein concentration and the occurrence of *Plasmodium* infection. We also saw that the concentration of tocopherol negatively explained the severity coccidiosis. It is known that *Isospora* sp. can damage the walls of the duodenum and intestine, leading to reduced absorption of vitamins in the intestinal tract (Giacomo *et al.*, 1997). Thus, house finches that had a higher severity of coccidia may absorb less tocopherol, and/or must be using the reserves to combat parasitism by this disease, which explains the negative relationship between coccidiosis severity and tocopherol. Finally, we found that males that had more circulating 3-hydroxy-echinenone appeared to have redder plumage. This result corroborates the important relationship of 3-hydroxy-equinenone as one of the most important pigments for red coloration for this species, and that males that manage to acquire rich resources with this carotenoid, manage to obtain a more reddish plumage.

Overall, our results demonstrate that dichromatic species are potential disease reservoirs and should be considered in projects that attempt to identify vulnerable species in conservation projects. Furthermore, adding the identification of parasites in ecological studies can help to better understand the physiological and morphological processes of species. Consequently, once we better understand how antagonistic interactions affect free-ranging bird populations, we can make better decisions about how to act to protect endangered species as well.

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